Second Edition

MICROBIOLOGY

Laboratory Theory & Application

ESSENTIALS

Lourdes Norman-McKay • Michael J. Leboffe • Burton E. Pierce

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This book is dedicated to the more than 3,000 U.S. health-care workers who lost their lives to COVID-19 during the 2020–2021 pandemic.

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PREFACE

Revision Reflections

The bulk of this second edition of *Microbiology: Laboratory Theory & Application, Essentials*, was developed under the specter of COVID-19. Given this timing, I often reflected on the word *essential*—a word that echoed in the media as we heard about essential workers, essential new vaccines, and essential treatments and transmission precautions. The fact that this book has been distilled to essentials from more comprehensive progenitors of the *Microbiology: Laboratory Theory & Application* series (*MLTA* series) was not lost on me, and it reminded me that most of the students taking an introductory microbiology course are interested in pursuing a career in health care-related fields. Indeed, it was a driving force for envisioning how to further refine the book to meet the training needs of our essential health-care workforce of tomorrow—or, as I refer to them, my students, many of whom are actively engaged in countermanding the pandemic.

My colleagues and now-retired peer authors, Mike Leboffe and Burt Pierce, spent the better part of three decades shaping the *MLTA* series and it has been my distinct honor to become the steward and new voice of the series. As Mike has done in previous revisions that he spearheaded upon Burt retiring, I maintained the "we" in the revision as a recognition of this work as an evolution of a longer standing and already warmly received text. My intentions in this revision were not to supplant these talented and experienced voices, but to augment them in a way that rounds out the series with an eye on molecular methods and medical applications, both of which are areas within my expertise as a medical scientist.

To that end, this second edition revision is a comprehensive reflection on the student laboratory learning experience and what is essential and employable for students interested in becoming health-care workers or pursuing medical microbiology, a laboratory sciences credential, or another medically focused discipline. The revision also considers those students who take a microbiology course to enter public health, epidemiology, or some other career where an introduction to microbiology laboratory curriculum is useful. Ultimately, at some point in our lives we are all patients, so a generalist non-major would also find this content useful. In short, this revision considers the student who is new to microbiology and may not yet have substantive prerequisite knowledge.

Synopsis of Changes from *MLTA Essentials*, 1e, to *MLTA Essentials*, 2e

1. The lab exercise order has been reorganized to more closely align with the order of a typical microbiology lecture/lab sequence.
2. Learning outcomes have been added to all exercises.
3. Content has been partitioned under more subheadings to decrease cognitive load.
4. Lab Technique reference sheets have been added to allow students to easily refer to them when performing these techniques in later labs.
5. The Background and Clinical Application sections were modified to better meet students where they are coming from and explain to them where they need to go and what they need to know to be successful in a clinical setting.
6. Wording in figure legends has been reduced and details have been layered directly into the art where appropriate.
7. Procedural diagrams now follow a linear format for easier comprehension.
8. Lab related questions have been diversified to include a more stratified representation of Bloom's learning taxonomy. In addition to the "in lab" documentation of findings, students now have access to multiple choice, true/false, labeling, short answer/fill in the blank, and critical thinking questions to support their review of content. These questions can be used as pre-lab and/or post-lab work.
9. Many references have been removed to save space. References have been added to footnotes only for material that is cutting edge and may not yet be widely known, for topics that are not general knowledge and that must really be investigated, and for hotly debated topics.

Exercise 1

- This exercise is divided into parts A, B, and C to help faculty more easily direct students to the parts of the lab they plan to cover.
- A specific exercise for using oil immersion was added.
- The discussion of how to calculate the limit of resolution was omitted.

- The discussion of numerical aperture also was omitted.
- A review of real versus virtual images and the optics details therein was deleted.

Exercise 2

- This exercise is divided into parts A, B, and C to help faculty more easily direct students to the parts of the lab they plan to cover.
- Materials for each section are cross-referenced for easier set-up.
- The "measuring organisms" aspect of the lab was removed, because the vast majority of reviewers stated that calibrating the micrometer is not a lab they deem essential, and many said they omit it. Also, quantitative assessment of microbial dimensions is not usually a learning outcome in an allied health microbiology course.
- The review of eukaryotic organisms by superkingdoms was eliminated to more closely mirror what students would be doing in the lecture portion of the course (the three domain and six kingdom model). In medicine, we still refer to protists and specifically to protozoans. This is important terminology for students to effectively tackle the pharmacology and pathophysiology aspects of their training. That said, the figure on the seven superkingdoms is retained and this taxonomic model's existence is acknowledged.
- A new figure on the superkingdoms was added.
- A revised figure on taxonomic domains and kingdoms also was added.

Exercise 3

- This exercise is divided into parts A, B, C, and D to help faculty more easily direct students to the parts of the lab they plan to cover.
- The procedural diagrams were completely overhauled to make the exercise easier for a novice to follow and perform.
- This lab now employs a highly modularized format in the form of Lab Techniques that give faculty the instructional flexibility they want and need.
- Inoculation of agar deeps was added. A high percentage of adopters use later labs that employ use of agar deeps and thus may appreciate an earlier exposure to how to handle agar deeps.
- Content on complex versus enriched media was added because we build on this concept in the biochemical test media section.
- A review of inoculating loops and needles and the commentary on aseptic transfer and its goals was incorporated into the lab instead of having it as a sidebar feature box before the lab.
- References to moving the tube versus the inoculating tool were removed. That approach is awkward in terms of dexterity maximization and anything that reduces manual dexterity is a risk in the lab.

Exercise 4

- This exercise is divided into parts A, B, and C to help faculty more easily direct students to the parts of the lab they plan to cover.
- The content was revised to make it more modularized.
- Additional content was added discussing environmental sampling with a strong cautionary statement on safety.
- Quadrant plate inoculation steps were combined into a single schematic (Fig. 4.3) and the text was modified to emphasize sterilization and cooling of the loop between plate sections, an essential step that students often overlook.
- The protocol was updated to include safer practices (e.g., keeping the lid on instead of exposing the sample to air).

Exercise 5

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 6

- The art layout was changed to a field guide format to highlight the beautiful photography.

Exercise 7

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 8

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 9

- A safety warning based on the CDC's safety recommendations for undergraduate teaching labs was added.

Exercise 10

- In this exercise, the explanations were simplified and calculations were broken down more systematically.
- Inherent limitations of the standard plate count method were included.

Exercise 11

- The photos were organized into a mini Instructional Atlas for Staining so that it can be highlighted as a key feature instead of relegated to a superfluous, between-the-exercise space.
- The lab was shifted to be a review of simple staining and bacterial cell morphology.
- The morphology content was incorporated into the lab proper to reinforce the message that the material is not optional or ancillary but is required to achieve the lab's teaching goal.
- Cell surface area content, which is more appropriate for a lecture, was removed.
- Staining screen was removed from the materials list, because it is not used in most teaching labs and isn't needed in the procedure.

Exercise 12

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 13

- The Background content for Gram-stain chemistry was clarified.
- A common misconception that students hold (i.e., that the Gram-staining procedure "makes" bacteria Gram positive or Gram negative) was addressed.
- The Gram-variability information was clarified.

Exercise 14

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 15

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 16

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 17

- The exercise title was modified to draw more attention to the exercise content because it is an important topic for introductory students.

Exercise 18

- Additional information on obligate anaerobes and also what to look for in the middle of the tube in terms of oxygen gas levels was added.

Exercise 19

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 20

- A new figure based on using 25 broth tubes instead of 21 was added (Fig. 20.3 broth and plate sample organization and inoculation process).

Exercise 21

- Terminology was revised to use the more prevalent term *neutralophile* to prevent student confusion between neutrophile and neutrophil.

Exercise 22

- A BSL-1 agent was added as a suggested replacement for the BSL-2 agent.
- Spot inoculation was omitted and replaced with zigzag inoculation because it is more successful in showing the desired effect.
- Table 22.1 was updated to remove common misconceptions.

Exercise 23

- The Background section was greatly expanded to explain what coliforms are and why we would want to be able to detect them in a sample.
- Updated information on neutral red dye was added to be more accurate.
- Spot inoculation was omitted and replaced with zigzag inoculation because it is more successful in showing the desired effect.
- Table 23.1 was updated to remove common misconceptions.

Exercise 24

- Spot inoculation was omitted and replaced with zigzag inoculation because it is more successful in showing the desired effect.
- Table 24.1 was updated to remove common misconceptions.

Exercise 25

- A safer option was added for professors who want to use stock cultures instead of throat swabs.
- For those using throat swabs, additional safety guidelines are provided.
- A correction was added regarding methemoglobin, which is oxidized, not reduced.
- A streak-stab technique for known stock cultures was included in the procedure to better align Background content with the procedure.

Exercise 26

- The Background section was entirely rewritten for clarity, including foundational details on the two metabolic processes mentioned. Electron tower content was removed.
- Content on ascertaining motility was removed because O-F media is not typically used for motility determination.

Exercise 27

- A key has been added to better explain fermentation broth results.
- Clarifying information has been added to Table 27.1 on fermentation broth test results and interpretations.

Exercise 28

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 29

- Extraneous content on how aerobic respiration generates toxic oxygen intermediates has been removed for a tighter alignment with lecture textbooks.
- Tables and figures have been more seamlessly interwoven into the Background section.

Exercise 30

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 31

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 32

- More information has been added about citrate, and corrections have been made to the mechanism involving citrate permease (citrate lyase acts before citrate permease; in addition, citrate permease is distinct from oxaloacetate decarboxylase).

Exercise 33

- A reminder to students to inoculate negative controls has been added.

Exercise 34

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 35

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 36

- Exercise 5-11 SIM Medium and Exercise 5-16 Motility Agar from the previous edition have been combined into one exercise. This was done because the stand-alone motility agar is usually only used when the bacterium reduces sulfur and thereby obscures the motility observations in SIM agar.

Exercise 37

- The content related to interpretations of the test results has been broken up to promote student comprehension.
- The explanation for the "reversion process" has been reworked to make it clearer for students who struggle with this topic.

Exercise 38

- The Application section has been greatly expanded to showcase why it is important to differentiate *S. aureus* from other less virulent *Staphylococcus* species and the problems that *S. aureus* can cause in the human body.

Exercise 39

- This is a new lab to the second edition of this book. It was added to showcase the importance of this test in identifying important pathogens such as *S. aureus*, *S. epidermidis*, *B. anthracis*, *B. cereus*, *C. tetani*, *C. perfringens*, *C. difficile*, and others.

Exercise 40

- A fuller definition and explanation of hand washing has been added to this lab since it is such a foundational tool for soon-to-be health-care providers.
- The procedure has been clarified so students know when to wear gloves and when not to.

Exercise 41

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 42

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 43

- The description of the zone of inhibition has been clarified, and students are more thoroughly instructed on how to develop conclusions about the test bacterium's susceptibility to the antibacterial drug(s) being tested.

Exercise 44

- Students are now encouraged to compare these tests to the Kirby-Bauer test performed in Exercise 42. In so doing, they will see that bacitracin, novobiocin, and optochin susceptibility testing is *far* less rigorous in terms of standardization than the Kirby-Bauer test. This is because bacitracin, novobiocin, and optochin susceptibility tests are not used to guide decisions about which drugs would be most effective against an infectious agent afflicting a patient. Instead, these susceptibility tests are for bacterial identification. The previous edition did not make this important point clear.
- This edition emphasizes to students that the drugs tested in this exercise are dangerous if ingested or injected and therefore, aside for certain topical applications, they have limited (and in some cases zero) approved applications in human medicine.

Exercise 45

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 46

- This epidemic simulation lab now has two versions: the first, as in the previous edition, uses *S. marcescens* as the transmissible agent; the second uses Glo Germ powder or gel as the transmissible agent. This will be a better option for schools with more stringent safety standards.

Exercises 47 and 48: Unknown Laboratory Exercise

The first edition included five unknown exercises. For this edition, these exercises were condensed down to two unknown labs (Ex. 47 and Ex. 48) and a variety of resources for aiding students as they perform the unknown exercises.

- Exercise 47: This dry lab helps students explore the process of identifying an unknown in the context of a clinical case. Because it is a dry lab it can be done in class or at home as either a preparation for the hands-on unknown lab exercise (Ex. 48) or as an augmentation to the wet-lab aspect of the course. The question series is deigned to be fully scorable and is carefully designed as a problem-based exploration to encourage students to carefully reflect upon and apply what they have learned.
- Exercise 48: The flowcharts from the previous edition still exist in an appendix. However, the content has been modified to better express to students that time is of the essence in clinical situations and that consequently a variety of biochemical tests are typically performed simultaneously to reduce time to definitive bacterial identification.

Acknowledgments

The first time I met Mike Leboffe in person was at an ASMCUE (American Society for Microbiology, Conference for Undergraduate Educators) meeting in Washington, DC, in 2019. Ahead of this in-person encounter, Mike and I mainly knew of one another through our respective authoring endeavors and through our conversations with David Ferguson, President and CEO of Morton Publishing. Knowing that Mike was ready to follow Burt into retirement, David brokered a year-long episode of the Dating Game to bring us together and map a path to *MLTA*'s continued legacy and evolution.

Having labored on developing and updating my own textbook, I can fully appreciate how, after so many years of attentive care, it would be difficult to entrust one's "book baby" to someone else. After all, a book used to teach *thousands* of people undergoes a considerable amount of scrutiny, and putting one's name alongside another's on the cover takes a tremendous amount of trust and vulnerability tolerance. As such, I sincerely thank Mike and Burt for their vote of confidence that equates to entrusting me to be the new captain of the *MLTA* ship they built and launched. I promise you both that I'll carefully circumvent icebergs when charting new waters, keep the rigging in good order, and not fall prey to the sirens of complacency as the discipline and teaching practices evolve.

As the top executive at Morton Publishing, David would have some difficult questions to answer if he selected the wrong person to assume one of Morton's flagship enterprises, so I am honored that he entrusted the task to me. All this to say, being the steward and new voice of the career work of others has a weighty significance that I am proud to undertake and do not take lightly. It's a tangible compliment be deemed "the right person."

For my part, taking on the *MLTA* series was not something I ventured into without serious reflection. Being an author is *a lot* of work and most academic authors, this one included, have numerous irons in the

proverbial fire. I had to consider how and if I could give the *MLTA* series the attention it requires while balancing my other career and familial obligations. The task wasn't without its challenges, but thanks to Marta Pentecost (Senior Acquisitions Editor) and Rayna Bailey (Editorial Project Manager), those challenges were made conquerable. These brilliant and impressive women made working closely while socially distancing as natural as breathing. Of course, the editorial effort would never have wings without the production team in our star lineup. I want to thank Will Kelley (Production Manager) and Joanne Saliger (Production Assistant) for their careful work on paging, implementing art and design revisions, and all other things production related.

I also extend my gratitude to our faculty reviewers, adopters, and students who have provided invaluable feedback to direct the revision process. You are the "why" behind it all. To Erin Amerman, my dear friend and fellow authoress, I owe a special debt of gratitude for her unflagging friendship, encouragement, and vote of confidence. I have not encountered many people who are as secure and grounded as Erin and who have the generosity of spirit that she has.

Lastly, I thank my much-loved family for their support as I spent *many* late nights and weekends entrenched in edits and page proofs. To my two cherished daughters, Lourdes Catherine and Delia, thank you for teaching me how to love unconditionally. And, to my dear husband, Andrew, you are the glue that keeps me together. ●

Lourdes Norman-McKay, Ph.D.
April 2021

About the Author

Lourdes Norman-McKay, Ph.D.

Dr. Norman-McKay earned her B.S. in microbiology and cell science from the University of Florida and her Ph.D. in biochemistry and molecular biology from the Pennsylvania State University College of Medicine. She completed a postdoctoral fellowship in microbiology and immunology at Penn State College of Medicine, where she was awarded a National Research Service Award from the National Institutes of Health to study the role of viruses in cancer. She has 19 years of experience teaching a wide range of general sciences such as biology, virology, biochemistry, microbiology, anatomy and physiology, and clinical genetics to students at all levels from freshman to post-graduates. She has been recognized by her peers and students with the Outstanding Faculty Award at Florida State College at Jacksonville, where she is currently a full-time professor. Dr. Norman-McKay has also served as an associate dean over science programs, developed curricula for STEM programs (to include a baccalaureate degree program in biomedical sciences and various STEM workforce certification programs), and served as a curriculum designer and subject matter expert for the Florida Space Research Institute. She is actively engaged as a section editor for the American Society for Microbiology's *Journal of Microbiology and Biology Education* and has been a central contributor to diverse STEM education outreach initiatives through ASM, to include drafting new learning outcomes for Microbiology in Nursing and Allied Health. She is also a speaker for the U.S. Department of State's Bureau of International Information Programs, through which she promotes innovation in STEM education and women in STEM. In addition to being the new lead author of the *Microbiology: Laboratory Theory and Application* series, she is also the author of *Microbiology: Basic and Clinical Principles*. In her free time, she enjoys traveling, reading, spending time with family, and exploring the great outdoors in Vermont. She lives in St. Johns, Florida, with her two daughters and husband.

Check out her TEDx talk, *The Love Affair with Hating Science.*

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— Lab Notes —

Health Hazard (Long-term)

Flammable

Irritant

Compressed Gas

Corrosive

Explosive

Oxidizer

Environmental Hazard

Toxic

Introduction: Safety in the Microbiology Laboratory

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Safety and Laboratory Guidelines

We hope that you find microbiology lab to be an interesting and exciting experience, but at the outset you must be made aware of some potential hazards. Improper handling of chemicals, equipment, and/or microbial cultures is dangerous and can result in injury or infection. Safety with lab equipment will be addressed when you first use that specific piece of equipment, as will specific examples of chemical safety. Our main concern here is to introduce you to safe handling and disposal of microbes.[1]

Because microorganisms present varying degrees of risk to laboratory personnel (oneself, other students, technicians, and faculty), people outside the laboratory, and the environment, microbial cultures must be handled safely. Classifying microbes into four biosafety levels (BSLs) provides a set of minimum standards for laboratory practices, facilities, and equipment to be used when handling organisms at each level. These biosafety levels, defined in the U.S. government publication, *Biosafety in Microbiological and Biomedical Laboratories*, 6th edition (2020), are summarized here and in Table S.1. For complete information, readers are referred to the original document.

[1] Your instructor may augment or revise these guidelines to fit the conventions of your laboratory.

BSL-1: Organisms do not typically cause disease in healthy individuals and present a minimal threat to the environment and lab personnel. Standard microbiological practices are adequate. These microbes may be handled in the open, and no special containment equipment is required. Examples include *Bacillus subtilis*, *Escherichia coli* (most strains), *Rhodospirillum rubrum*, and *Lactobacillus acidophilus*.

BSL-2: Organisms are commonly encountered in the community and present a moderate environmental and/or health hazard. These organisms are associated with a variety of human diseases, most of which can be successfully treated if identified in a timely manner. The infection routes of primary concern are ingestion, inhalation, or penetration of the skin (percutaneous). Individuals performing work prone to splashes or aerosol generation (even though these organisms are not generally known to be transmitted by aerosols) should work in a biological safety cabinet (BSC, Fig. S.1). Otherwise, laboratory work may be done using standard microbiological practices. Examples include *Salmonella*, *Staphylococcus aureus*, *Clostridioides difficile* (formerly *Clotsridium difficile*), and *Borrelia burgdorferi*.

Biosafety Cabinets

Biosafety cabinets are classified into three categories, all of which draw air into the cabinet to minimize microbial contamination back into the room.

Class I BSCs resemble chemical fume hoods with a protective glass in the front, but have a HEPA filter along the exhaust path to prevent microbes from entering the environment.

Class II BSCs use laminar airflow, which minimizes turbulence, and HEPA filters to protect the user and materials within the cabinet from contamination. The exhaust passes through a HEPA filter before its release into the environment.

Class III BSCs are completely sealed and gas tight with a fixed viewing window. Incoming air passes through a HEPA filter, whereas materials to be handled are passed through a double-door system where the intermediate compartment is an autoclave. (Other methods may also be used, but accomplish the same purpose.) Exhaust air passes through two HEPA filters or a HEPA filter and an air incinerator. Materials are handled with arm-length, heavy-duty gloves built into the wall of the cabinet. ●

FIGURE **S.1 Biological Safety Cabinet (BSC) in a Teaching Laboratory** ● In this Class II BSC, air is drawn in from the room and is passed through a HEPA filter prior to release into the environment. This airflow pattern is designed to keep aerosolized microbes from escaping from the cabinet. The microbiologist is pipetting a culture. When the BSC is not in use at the end of the day, an ultraviolet light is turned on to sterilize the air and the work surface.

BSL-3: Organisms are of local or exotic origin and are associated with respiratory transmission and serious or lethal diseases where treatment and/or vaccines may or may not be available. Special ventilation systems are used to prevent aerosol transmission out of the laboratory, and access to the lab is restricted. Specially trained personnel handle microbes in a Class II or III BSC, not on the open bench. Examples include *Bacillus anthracis*, *Mycobacterium tuberculosis*, and West Nile virus.

BSL-4: Organisms have a great potential for lethal infection. Inhalation of infectious aerosols, exposure to infectious droplets, and autoinoculation are of primary concern. The lab is isolated from other facilities, and access is strictly controlled. Ventilation and waste management are under rigid control to prevent release of the microbial agents to the environment. Specially trained personnel perform transfers in Class III BSCs. Class II BSCs may be used as long as personnel wear positive pressure, one-piece body suits with a life-support system. Examples include agents causing hemorrhagic diseases, such as Ebola, Marburg, and Lassa fever viruses.

The microorganisms used in introductory microbiology courses depend on the institution, objectives of the course, and student preparation. Most introductory courses use organisms that may be handled at BSL-1 and BSL-2 levels, so we have followed that practice in designing this set of exercises.

Following are general safety rules to reduce the chance of injury or infection to you and to others, both inside and outside the laboratory. Although both BSL-1 and BSL-2 guidelines are listed, we believe it is best to err on the side of caution and that students should learn and practice the safest level of standards (relative to the organisms they are likely to encounter) at all times. Please follow these and any other safety guidelines required by your college.

Chemical safety is also important in a microbiology laboratory. Be aware of the hazards presented by the chemicals you are handling. Containers should be labeled with a standard set of precautions as seen in Figure S.2. Numbers are assigned to the degree of health, fire, and reactivity hazard posed by the chemical. On stickers placed by the laboratory, there also is a space to enter specific hazards, such as acid, corrosive, and radioactivity. Pictograms, shown at the beginning of this section, are also used.

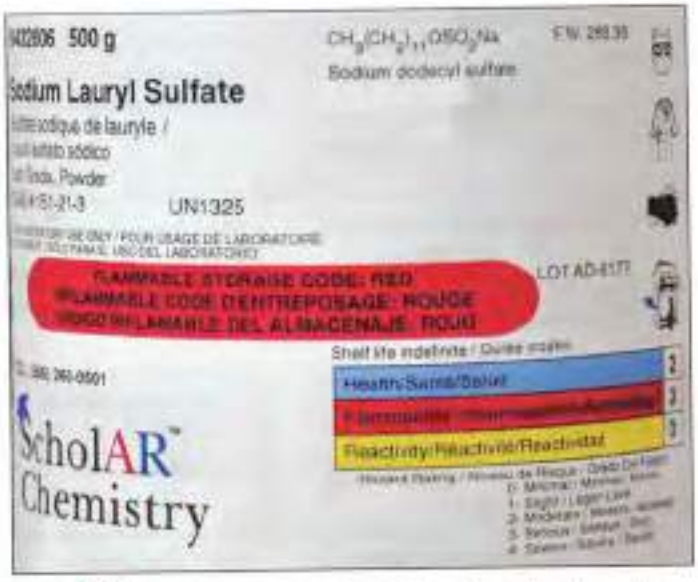


FIGURE **S.2** **Chemical Hazard Label** • The blue, red, and yellow boxes on this label indicate the health, flammability, and reactivity levels, respectively, of the compound sodium dodecyl sulfate (SDS). The colors are standardized (regardless of the chemical) and each is assigned a number between "0" (minimal) and "4" (severe) as shown in the legend at the lower right. SDS presents a moderate health hazard, is very flammable, but minimally reactive. Also notice the icons in the upper right, which indicate (from top to bottom) that this compound should be handled with eye protection, a lab coat, gloves, and adequate ventilation. Be aware of these warnings and comply with the associated cautions for all chemicals you handle in lab.

TABLE **S.1** Summary of Recommended Biosafety Levels for Infectious Agents •

BSL	Agents	Practices	Safety Equipment (primary barriers)	Facilities (secondary barriers)
1	▪ Not known to consistently cause disease in healthy adults ▪ e.g., *Lactobacillus casei*, *Bacillus subtilis*, *Rhizobium leguminosarum*	▪ Standard microbiological practices	▪ Primary barriers: none required ▪ Personal Protective Equipment (PPE): laboratory coats and gloves; eye, face protection as needed	▪ Laboratory bench practices and sink required
2	▪ Agents associated with human disease; treatments and/or vaccines are usually available ▪ Routes of transmission include percutaneous injury, ingestion, and mucous membrane exposure ▪ e.g., *Staphylococcus aureus*, *Salmonella enterica*, measles virus	**BSL-1 practices plus:** ▪ Limited lab access ▪ Biohazard warning signs ▪ "Sharps" precautions ▪ Biosafety manual defining any needed waste decontamination	▪ Primary barriers: Class I or II BSCs or other physical containment devices used for all manipulations of agents that cause splashes or aerosols of infectious materials ▪ PPEs: laboratory coats, gloves, face protection as needed	**BSL-1 plus:** Autoclave available
3	▪ Indigenous or exotic agents with potential for aerosol transmission ▪ Disease may have serious or lethal consequences; treatments and/or vaccines may be available ▪ e.g., *Yersinia pestis*, *Mycobacterium tuberculosis*, rabies virus	**BSL-2 practices plus:** ▪ Controlled lab access ▪ Decontamination of all waste ▪ Decontamination of all lab clothing before laundering ▪ Baseline serum	▪ Primary barriers: Class I or II BSCs or other physical containment devices used for all manipulations of agents ▪ PPEs: protective lab clothing, gloves, respiratory protection as needed	**BSL-2 plus:** ▪ Physical separation from access corridors ▪ Access to self-closing double door ▪ Exhausted air not recirculated ▪ Negative airflow into laboratory
4	▪ Dangerous/exotic agents that pose high risk of life-threatening disease, aerosol-transmitted lab infections, or related agents with unknown risk of transmission; treatment and vaccines are unavailable ▪ e.g., Ebola virus, Lassa virus	**BSL-3 practices plus:** ▪ Clothing change before entering ▪ Shower on exit ▪ All material decontaminated on exit from facility	▪ Primary barriers: all procedures conducted in Class III BSCs or Class I or II BSCs *in combination with* full-body, air-supplied, positive pressure personnel suit	**BSL-3 plus:** ▪ Separate building or isolated zone ▪ Dedicated supply and exhaust vacuum, and decon system ▪ Other requirements outlined in the text

Source: Adapted from *Biosafety in Microbiological and Biomedical Laboratories*, 6th edition (Washington, DC: U.S. Government Printing Office, 2020).

Student Conduct

- To reduce the risk of infection, do not smoke, eat, drink, or bring food or drinks into the laboratory room, even if lab work is not being done at the time.
- Do not apply cosmetics or handle contact lenses in the laboratory.
- Wash your hands *thoroughly* with soap and water after handling living microbes and before leaving the laboratory each day. Also, wash your hands after removing gloves.
- Do not remove any organisms or chemicals from the laboratory.
- Lab time is precious, so come to lab prepared for that day's work. Figuring out what to do as you go is likely to produce confusion and accidents.
- Work carefully and methodically. Do not hurry through any laboratory procedure.

Basic Laboratory Safety

- Wear protective clothing (i.e., a lab coat) in the laboratory when handling microbes. Remove the coat prior to leaving the lab and autoclave it regularly (Fig. S.3). Do not take it home for washing until it has been autoclaved.
- Wear only closed-toe shoes in the laboratory.
- Wear eye protection whenever you are handling microbes or chemicals (especially during heating) even if you wear glasses or contacts (Fig. S.3).
- Turn off your Bunsen burner when it is not in use. In addition to being a fire and safety hazard, it is an unnecessary source of heat in the room.
- Tie back long hair, because it is a potential source of contamination as well as a likely target for fire.
- If you are feeling ill, go home. A microbiology laboratory is not a safe place if you are ill.
- If you are pregnant, immune compromised, or are taking immunosuppressant drugs, please see the instructor. It may be in your best long-term interests to postpone taking this class. Discuss your options with your instructor.
- If it is your lab's practice to wear disposable gloves while handling microorganisms (and this is becoming the norm), be sure to remove them each time you leave the laboratory. The proper method for removal is with the thumb under the cuff of the other hand's glove and turning it inside out without snapping it (Fig. S.4). Gloves should then be disposed of in the container for contaminated materials. Finally, wash your hands.
- Wear disposable gloves while staining microbes and handling blood products—plasma, serum, antiserum, or whole blood. Handling blood can be hazardous, even if you are wearing gloves. Consult your instructor before attempting to work with any blood products.
- Use an antiseptic (e.g., Betadine) on your skin if it is exposed to a spill containing microorganisms. Your instructor will tell you which antiseptic you will be using. Have a lab partner turn on the water for you if your hands have been contaminated.
- Never pipette by mouth. Always use a mechanical pipettor (Fig. C.1, Appendix C).
- Dispose of broken glass or any other item that could puncture an autoclave bag (contaminated or not) in an appropriate sharps or broken-glass container (Fig. S.5).
- Use a fume hood to perform any work involving highly volatile chemicals or stains that need to be heated.
- Find the first-aid kit, and make a mental note of its location.
- Find the fire blanket, shower, and fire extinguisher, note their locations, and develop a plan for how to access them in an emergency.
- Find the eyewash basin, learn how to operate it, and remember its location.

Reducing Contamination of Self, Others, Cultures, and the Environment

- Wipe the desktop with a disinfectant (e.g., Coverage or 10% chlorine bleach) before *and* after each lab period. Never assume that the class before you disinfected the work area. An appropriate disinfectant will be supplied. Allow the disinfectant to evaporate; do not wipe it dry.

FIGURE **S.3** **Safety First** • This student is prepared to work safely with microorganisms. The lab area is uncluttered, tubes are upright in a test-tube rack, and the flame is accessible but not in the way. The student is wearing a protective lab coat, gloves, and goggles, all of which are to be removed prior to leaving the laboratory. Not all procedures require gloves and eye protection. Your instructor will advise you as to the standards in your laboratory.

FIGURE S.4 **Proper Glove Removal** • Protective gloves fit tightly around the hands and must be removed in such a way that limits their snapping, which produces aerosols. (A) Begin by hooking the thumb of one hand under the cuff of the glove on the other hand. (B) Next, roll the glove off the hand so that it turns inside out. (C) Take the removed glove in the hand that still has a glove on it and repeat the process. (D) As you roll the second glove off your hand, the first glove will end up inside the second glove, which also ends up inside out. Upon completion, all contaminated surfaces are inside the second glove.

- Never lay culture tubes on the table; they always should remain upright in a tube holder (Fig. S.3). Even solid media tubes contain moisture or condensation that may leak out and contaminate everything it contacts.
- Cover any culture spills with paper towels. Soak the towels immediately with disinfectant and allow them to stand for 20 minutes. Report the spill to your instructor. When you are finished, place the towels in the container designated for autoclaving.
  - If the culture spills on you, remain where you are, do not touch anything, and have your lab partner notify the instructor. Your instructor will advise your group on how to handle the spill.
  - If you get a microbial culture in your eyes, IMMEDIATELY have a lab partner lead you to the eyewash basin and rinse your eyes for at least 15 minutes. Time is of the essence for eye contamination. Your lab partners must notify the instructor of the spill and your situation, and follow their instructions for handling your care and the spill.
- Keep all nonessential books and papers off the desk. A cluttered lab table is an invitation for an accident that may contaminate your expensive school supplies. Your instructor will advise you on where to store these items.
  - Cell phones, tablets, computers, and other electronic devices must never be on the lab table when working with microbes. Contamination of these items can be a health hazard as well as expensive.

FIGURE S.5 **Sharps Container** • Needles, glass, and other contaminated items that can penetrate the skin or an autoclave bag should be disposed of in a sharps container. Do not fill above the dashed black line. Notice the autoclave tape in the lower left. The white stripes will turn black after proper autoclaving. Above the autoclave tape is the address of the institution that produced the biohazardous waste.

- When pipetting microbial cultures, place a disinfectant-soaked towel on the work area. This reduces contamination and possible aerosols if a drop escapes from the pipette and hits the tabletop.

Guidelines Governing Handling of BSL-2 Organisms

Following is a list of precautions for handling BSL-2 organisms. A number of these were covered previously, but are repeated here along with the new ones and now are considered "highly recommended." It is also highly recommended that before students are allowed to handle BSL-2 organisms, they should demonstrate skill in handling BSL-1 organisms safely.

Your college may have other guidelines for handling BSL-2 organisms and other standards of practice may be adopted during the lifetime of this book, so pay attention to any announcements your lab instructor may give. And, as always, it is the instructor's responsibility to choose organisms and lab exercises appropriate to the skill level of their students.

- The lab instructor should announce to the class when BSL-2 precautions are in effect. Your college may also institute a mechanism for identifying BSL-2 cultures, such as using red caps on culture tubes. Be aware of any conventions used by your lab.
- Access to the lab must be limited once work with the BSL-2 organisms has begun. Doors and windows must be closed.
- All unnecessary materials (books, backpacks, etc., in other words, anything you plan on taking home) must be kept off the work surface and safe from aerial contamination and spills. Your lab will have a designated place for their storage.
- Disinfect your lab tabletop before and after the activity. Allow the disinfectant to air-dry.
- A lab coat, gloves, and eye protection must be worn throughout the handling of BSL-2 organisms. Do not touch any items not directly involved in the lab activity. Properly remove and dispose of the gloves when finished. (See Fig. S.4 for proper glove removal.)
- Writing utensils must be "lab dedicated," that is, they do not leave the lab until they have been disinfected. It is best to wipe them down with disinfectant after use but keep them in your lab drawer for the entire semester. There is no sense in risking contamination of multiple pens and pencils by using different ones each day.
- Electronic devices (e.g., laptops, tablets, cell phones) must be kept off the work surface and stored in an area protected from spills and aerial contamination.
- Use page covers to protect your lab exercise pages while performing the activity. Once your work is completed, disinfect the page covers, wash your hands, and remove the pages. When dry, the page covers can be stored in your locker and reused. The lab book pages can be taken home.
- It is best to use a photocopy of your data sheet to record results. Once you have decontaminated the area, it can be photographed or scanned with a dedicated document scanner in the lab and the results can be transcribed to the original data sheet. Dispose of the photocopy in an appropriate biohazard container. It must not leave the lab.
- Minimizing aerosol production during the transfer of microorganisms is important and the instructions in Section 1 address methods for doing so. However, here is a summary of recommended practices specific to BSL-2 organisms.
  - Wire loops and needles should be incinerated in an enclosed electric incinerator (refer to Section 3, Fig. 3.3A, p. 51). Sterilization with an open flame should be avoided because of its potential for aerosol production.
  - An alternative to the electric incinerator is to perform transfers with sterile disposable loops or sterile wooden sticks. Both will work for transferring from solid or liquid media. After each use, they should be put in a disposal container (such as a can) contaminated end down, submerged in about 2 cm of disinfectant. When finished with the exercise, the can should be put in a bin for autoclaving, or any other disposal receptacle your lab uses. Alternatively, sticks and disposable loops can be put in a sharps container if that is your lab's practice.
  - Pipetting must be done in such a way that the last drop is *not* "blown out" unless a petri plate lid is covering a plate. Appendices C and D give specific instructions for pipetting BSL-2 organisms.
- Clean up after the activity.
  - For incubation, place tubed cultures in a rack. For plate cultures, it is best to tape the lids on each plate individually by taping around the edge of the plate rather than taping around the base and lid (Fig. S.6). This allows viewing of the agar surface without having to remove the tape.
  - Wipe down the work surface, writing utensils, and page covers (if any) with disinfectant and let them air-dry. Only after the disinfectant has dried is it okay to place items to be removed from lab on your table.

- Remove your gloves, eye protection, and lab coat and store them according to your lab's conventions.
- Wash your hands thoroughly.

- Photograph or scan your data sheet and store or dispose of it properly.

Disposing of Contaminated Materials

In most instances, the preferred method of decontaminating microbiological waste and reusable equipment is the autoclave (Fig. S.7).

- Remove all labels from tube cultures and other contaminated *reusable* items and place them in the designated autoclave container. This will likely be an open autoclave pan to enable cleaning the tubes and other items following sterilization.
- Dispose of plate cultures (if plastic petri plates are used) and other contaminated non-sharp *disposable* items in the designated autoclave container, such as an autoclave bag (Fig. S.8). Petri plates should be taped closed. (*Note:* Autoclave containers are designed to be autoclaved, permanently closed, and discarded. Therefore, do not place reusable and nonreusable items in the same container.)

FIGURE S.6 **Taping Plates** • It is a good idea to tape the lid of a petri plate to its base when incubating or storing it, especially if it contains a BSL-2 organism or a culture of unknown microbes. (**A**) One method is to wrap a length of tape around the lid and then use your thumb to press it firmly to the base. (**B**) When finished, the entire agar surface is visible and not obscured by any tape, making lid removal unnecessary to view colonies.

FIGURE S.7 **An Autoclave** • Media, cultures, and equipment to be sterilized are placed in the basket of the autoclave. Steam heat at a temperature of 121°C (produced at atmospheric pressure plus 15 psi) for 15 minutes is effective at killing even bacterial spores. Some items that cannot withstand the heat, or have irregular surfaces that prevent uniform contact with the steam, are sterilized by other means.

FIGURE S.8 **An Autoclave Bag** • Nonreusable items (such as plastic petri plates) are placed in an autoclave bag for decontamination. petri plates should be taped closed. Do not overfill or place sharp objects in the bag. Notice the autoclave tape at the middle right. The white stripes will turn black after proper autoclaving. At the lower right is the address of the institution that produced the biohazardous waste.

- Dispose of all blood product samples and disposable gloves in the container designated for autoclaving.
- Place used microscope slides of bacteria in a sharps container designated for autoclaving, or soak them in disinfectant solution for at least 30 minutes before cleaning or discarding them. Follow your laboratory guidelines for disposing of glass.
- Place contaminated broken glass and other sharp objects (anything likely to puncture an autoclave bag) in a sharps container designated for autoclaving (Fig. S.5). Uncontaminated broken glass does not need to be autoclaved, but should be disposed of in a specialized, broken-glass container.

Student Compliance with Laboratory Safety Regulations

Your institution may augment the safety regulations outlined above or compile a list tailored to your laboratory. In either case, these constitute a Laboratory Safety Statement. It is highly recommended that students verify that they have read and understand these safety regulations, agree to comply with them, and are aware of noncompliance consequences by signing a statement to that effect. The American Society for Microbiology has produced a publication (Emmert et al., 2012) that includes a sample Laboratory Safety Statement and a student signature page. The signature portion in a slightly modified form is reproduced on page 13. Your instructor may have you sign it or sign their own version of it.

Reference

Meechan, P. J. and J. Potts. U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories*, 6th ed. Washington, DC: U.S. Government Printing Office, 2020.

A Word About Experimental Design

Like most sciences, microbiology has descriptive and experimental components. Here we are concerned with the latter. Science is a philosophical approach to finding answers to questions. Despite what you may have been taught in grade school about *THE* "Scientific Method," science can approach problems in many ways, rather than in any *single* way. The nature of the problem, personality of the scientist, intellectual environment at the time, and good, old-fashioned luck all play a role in determining which approach is taken. Nevertheless, in experimental science, one component that is always present is a **control** (or controls).

A controlled experiment is one in which all **variables** except one—the **experimental variable**—are maintained without change. Frequently, we are looking for the cause of some phenomenon. By limiting variables to only one, we can draw a provisional conclusion about whether or not that variable causes the phenomenon.

If changing the variable causes a change in the phenomenon, then we can provisionally conclude that the variable and the phenomenon are causally related; that is, we have demonstrated a **cause and effect relationship** between the phenomenon and the experimental variable. Alternatively, if there is no observed change, we can eliminate the experimental variable from involvement with the phenomenon.

For example, if we want to determine the effect of increasing temperature on microbial growth rate, we could grow the same microorganism in two test tubes containing the same nutrient source at two different temperatures. One would be grown at "normal" temperature (whatever it is for that organism) and the other would be grown at a higher temperature. Everything but temperature would be the same, so if we see a difference in growth rate we can attribute it to the experimental variable, temperature.

But how would we know there is a difference in growth rate? Simple, by comparing the growth rate at the higher temperature to the growth rate at the "normal" temperature. The "normal" temperature tube is the control; it provides a baseline growth rate against which the experimental growth rate is compared. Growth rate in the experimental tube will be faster, slower, or the same as the control.

Without the control we would only be able to measure growth rate in the experimental tube, but we couldn't answer the question about the effect of higher temperature on growth rate. Had we grown different microorganisms with different nutrient sources at different temperatures and we saw a difference, we wouldn't know if the difference was due to the organism, the nutrients available, and/or the temperature. This is why we test one variable at a time.

Controls are an essential and integral part of all experiments, but there are many types. Two commonly used controls are **positive** and **negative controls.** A positive control is one that is set up to produce a positive result. Alternatively, a negative control is set up to demonstrate a negative result or no change.

As you work your way through the exercises in this book, pay attention to the various ways positive, negative, and other controls are used to improve the **validity** (effectiveness) of the experiment and the **reliability** (accuracy) of the results. In fact, there are many questions on the data sheets that ask some form of the question: "What is the role of the control in this experiment?" We are pretty sure that your professor won't be satisfied with the answer, "It's a control."

Microbiological experimentation often involves tests that determine the ability of an organism to use or produce some chemical, or to determine the presence or absence of a specific organism in a sample. Ideally, a positive result in the test indicates that the microbe has the ability or is present in the sample, and a negative result indicates a lack of that ability or absence in the sample (Fig. S.9).

The tests we run, however, have limitations and occasionally may give **false-positive** or **false-negative** results. An inability to detect small amounts of the chemical or organism in question would yield a false-negative result and would be the result of inadequate **sensitivity** of the test (Fig. S.9). An inability to discriminate between the chemical or organism in question and similar chemicals or organisms would yield false-positive results when the similar chemical or organism was being tested and would be the result of inadequate **specificity** of the test (Fig. S.9). Sensitivity and specificity can be quantified using the following equations:

$$\text{Sensitivity} = \frac{\text{True Positives}}{\text{True Positives} + \text{False Negatives}}$$

$$\text{Specificity} = \frac{\text{True Negatives}}{\text{True Negatives} + \text{False Positives}}$$

The closer sensitivity and specificity are to a value of one, the more useful the test. As you perform the tests in this book, be mindful of each test's limitations, and be open to the possibility of false-positive and false-negative results.

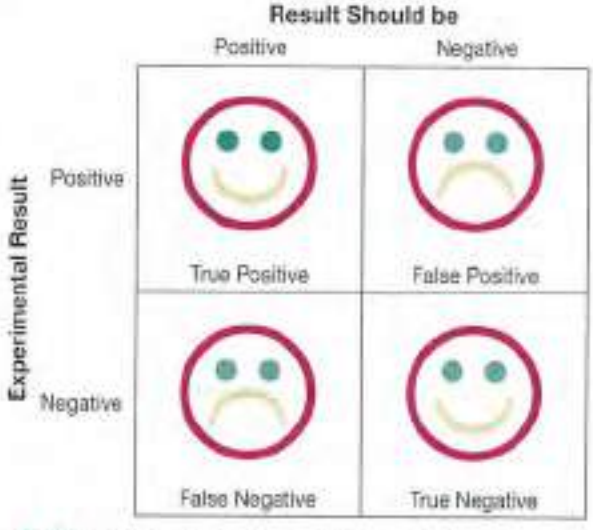


FIGURE **S.9** **Limitations of Experimental Tests** • Ideally, tests should give a positive result for specimens that are positive, and a negative result for specimens that are negative. False-positive and false-negative results do occur, however, and these are attributed to inadequate specificity and inadequate sensitivity, respectively, of the test system.

Data Presentation: Tables and Graphs

In microbiology, we perform experiments and collect data, but it is often difficult to know what the data mean without some method of organization. Tables and graphs allow us to summarize data in a way that makes interpretation easier.

Tables

A table is often used as a preliminary means of organizing data. As an example, Table S.2 shows the winning times for each male and female age division in a half-marathon race. Again, the aim of a table is to provide information to the reader. Notice the meaningful title, the column labels, and the appropriate measurement units. Without these, the reader cannot completely understand the table and your work will go unappreciated! Data tables are provided for you on the data sheets for each exercise in this book, but you may be required to fill-in certain components (units, labels, etc.) in addition to the data.

TABLE **S.2** **Winning Half-Marathon Times by Sex and Age Division** •

Male Runners		Female Runners	
Winner's Age (years)	Winning Time (minutes)	Winner's Age (years)	Winning Time (minutes)
15	73	15	88
25	67	24	82
31	67	30	82
35	71	39	84
40	71	42	85
52	78	50	109
62	95	62	108
70	123	70	126

Graphs

Table S.2 does give the information, but what it is telling us may not be entirely clear. It appears that the half-marathon times increase as the runners get older, but we have difficulty determining if this is truly a pattern. That is why data also are presented in graphic form at times; a graph usually shows the relationship between variables better than a table of numbers.

X–Y Scatter Plot The type of graph you will be using in this manual is an "X–Y Scatter Plot," in which two variables are graphed against each other. Figure S.10A shows the same data as Table S.2, but in an X–Y Scatter Plot form.

Notice the following important features of the graph in Figure S.10A:

- *Title:* The graph has a meaningful title, which should tell the reader what the graph is about. A title of "Age vs. Winning Time" is vague and inadequate.
- *Dependent and independent variables:* The graph is read from left to right. In our example, we might say for the male runners, "As runners get older, winning times get longer." *Winning time* depends on *age*, so winning time is the *dependent* variable and age is the *independent* variable. (Age does *not* depend on the winning time!) By convention, the independent variable is plotted on the *x*-axis and the dependent variable is plotted on the *y*-axis. By way of comparison, notice the consequence of plotting age on the *y*-axis and winning time on the *x*-axis: "As runners get slower, they get older," which doesn't reflect the actual relationship between the variables and worse yet, is nonsense.
- *Axis labels:* Each axis is labeled, including the appropriate units of measure. "Age" without units is meaningless. Does the scale represent months? Years? Centuries?
- *Axis scale:* The scale on each axis is uniform. The distance between marks on the axis is always the same and represents the same amount of that variable. (But increments on the *x*-axis don't have to equal those on the *y*-axis, as shown.) The size of each increment is up to the person making the graph and is dictated by the magnitude and range of the data. Most of the time, we choose a length for the axis that fills the available graphing space.
- *Axis range:* The scale for an axis does not have to begin with "0." Use a scale that best presents the data. In this case, the smallest *y*-value was 67 minutes, so the scale begins at 60 minutes.
- *Multiple data sets and the legend:* The two data series (male and female times) are plotted on the same set of axes, but with different symbols that are defined in the legend at the right. The symbols shown differ in color *and* shape, but one of these is adequate.
- *Best-fit line:* If a line is to be drawn at all, it should be an average line for the data points, not one that "connects the dots" (Fig. S.10B). Notice that the points are not necessarily *on* the line. The purpose of a best-fit line is to illustrate the general trend of the data, not the specifics of the individual data points. (Be assured that most graphs in your textbooks where a smooth line is shown were experimentally determined and the lines are derived from points scattered around the line.) There is a mathematical formula that allows one to compute the slope and *y*-intercept of the **trend line** if the relationship is linear, or a **best-fit line** if the relationship is nonlinear (as in the half-marathon times example), but this is beyond our needs. For our purposes, a hand-drawn trend line that looks good is good enough. (If you use a computer graphing program, then it will produce the trend line without you doing any of the math—the best of all situations!)

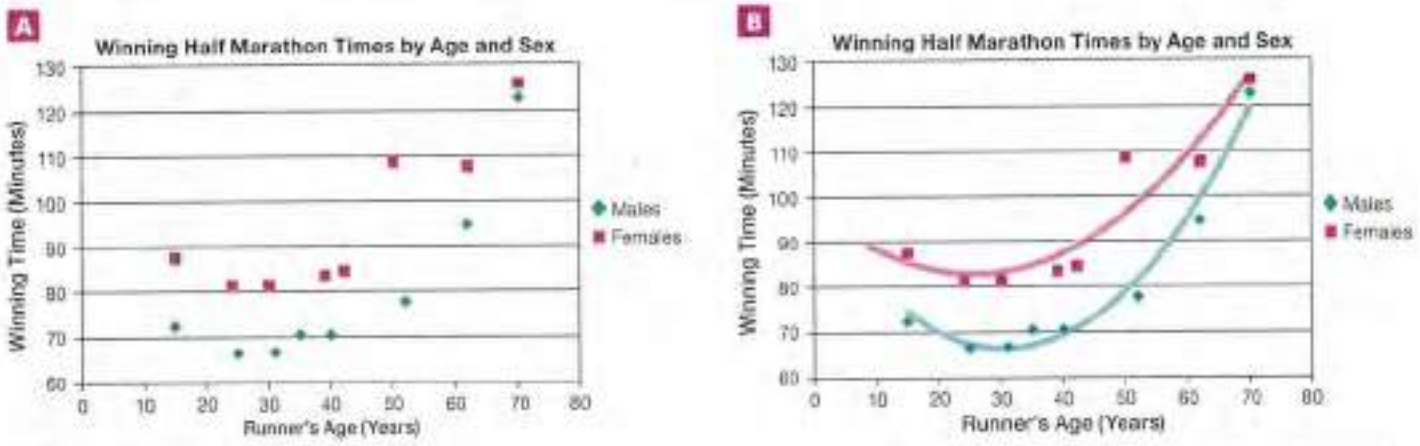


FIGURE S.10 Sample X-Y Scatter Plot • A graph often shows the relationship between variables better than a table of numbers. Examine this sample and identify the essential components of a quality graph (see text). (**A**) Presentation of data without a best-fit line is acceptable if there are not enough data points to justify illustrating a trend. (**B**) Shown here are the same data but with a trend line. Notice that the points do not fall directly on the line but, rather, that the line gives the general trend of the data. "Connecting the dots" is not appropriate.

Bar Graphs

Bar graphs are used to illustrate one variable. Often, the variable is considered to be categorical. That is, the data are in distinct groups (rather than continuous, where the breaks are arbitrary). Using a bar graph to show the relationship between winning times and ages is inappropriate. Examine Figure S.11A. Notice that the space each bar fills is meaningless; that is, the only important part of the bar is the top, which is the value used in the *X*–*Y* scatter plot. Also notice that the *x*-axis scale is not uniform—the gaps between bars range from 4 years (31 to 35) to 12 years (40 to 52). An appropriate use of a bar graph would be the distribution of student performance on an exam, even though exam scores are not categorical (Fig. S.11B). Notice that the space each bar fills has meaning. Each student in a particular group adds height to the bar.

Data Presentation: Be Creative, But Complete

There is no single correct way to produce a graph for a particular data set. Actually, most people working independently would graph the same data set in different ways (e.g., different scales, colors, wording of the title, and axis labels), but the essential components listed would have to be there. You will be asked to graph some of the data you collect. Be sure your graphs tell a complete and clear story of what you have done.

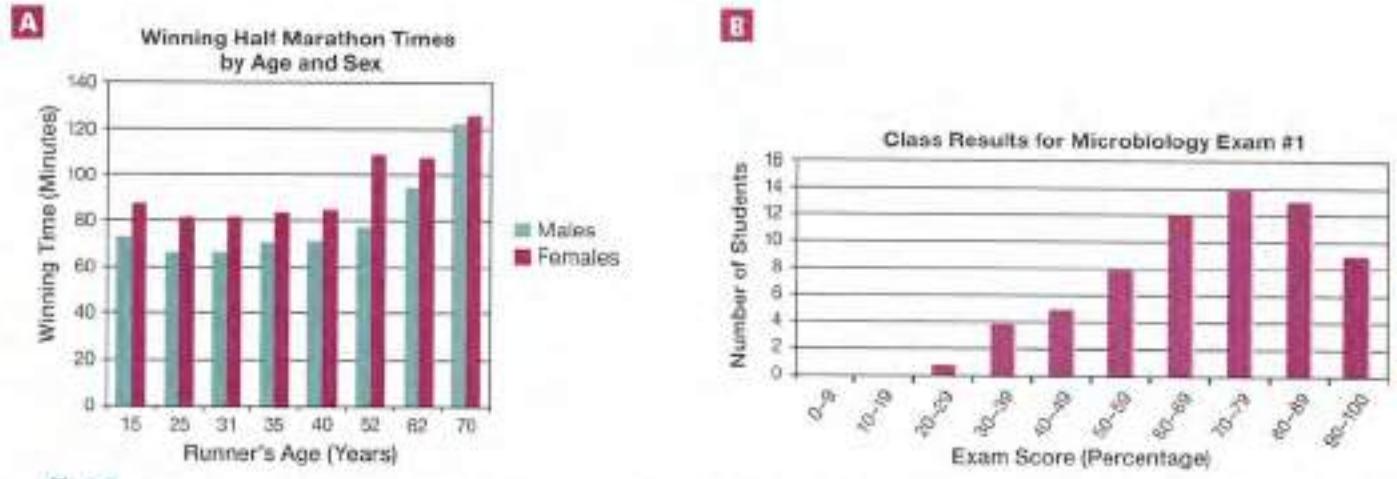


FIGURE S.11 Bar Graphs • A bar graph is appropriate to present data involving a single variable, especially if that variable is easily divided into distinct categories or groups. (A) Plotting the winning half-marathon times from Table 5.2 using a bar graph is inappropriate because the only meaningful point is at the top. (B) A bar graph is useful in presenting data of a single variable, such as the number of students earning a specific score on their microbiology exam.

STUDENT SAFETY CONTRACT

Student Agreement on Laboratory Safety

I have read the Laboratory Safety Statement as written in *Microbiology: Laboratory Theory & Application, Essentials*, 2nd edition, and/or the equivalent document supplied by the Department of Biological Sciences,

_____,

(Institution Name)

and I understand its content. I agree to abide by all laboratory rules set forth by the instructor. I understand that my safety is entirely my own responsibility and that I may be putting myself and others in danger if I do not abide by all the rules set forth by the instructor.

COURSE: _____

STUDENT NAME (PRINT): _____

STUDENT SIGNATURE: _____

DATE: _____

SECTION

1

Microscopy

In This Section

EXERCISE 1 Introduction to Light Microscopy 17

EXERCISE 2 Microscopic Examination of Eukaryotic Microbes 29

— Lab Notes —

EXERCISE 1

Introduction to Light Microscopy

After doing this exercise, you should be able to:

1. Identify and describe the functions of the compound light microscope's parts.
2. Perform bright-field microscopy to view specimens.
3. Use the high-power oil-immersion lens to view prepared specimens.
4. Calculate final magnification.
5. Name three other examples of light microscopy aside from bright-field microscopy.
6. Discuss light microscopy's clinical applications.

Background

There are many forms of microscopy, but **bright-field microscopy** is the simplest and most common way we observe microscopic organisms in the microbiology laboratory. Typically, we use a compound microscope for bright-field microscopy (Fig. 1.1). In bright-field microscopy, the specimen appears as a darker object set against a bright background—hence the technique's name (Fig. 1.2).

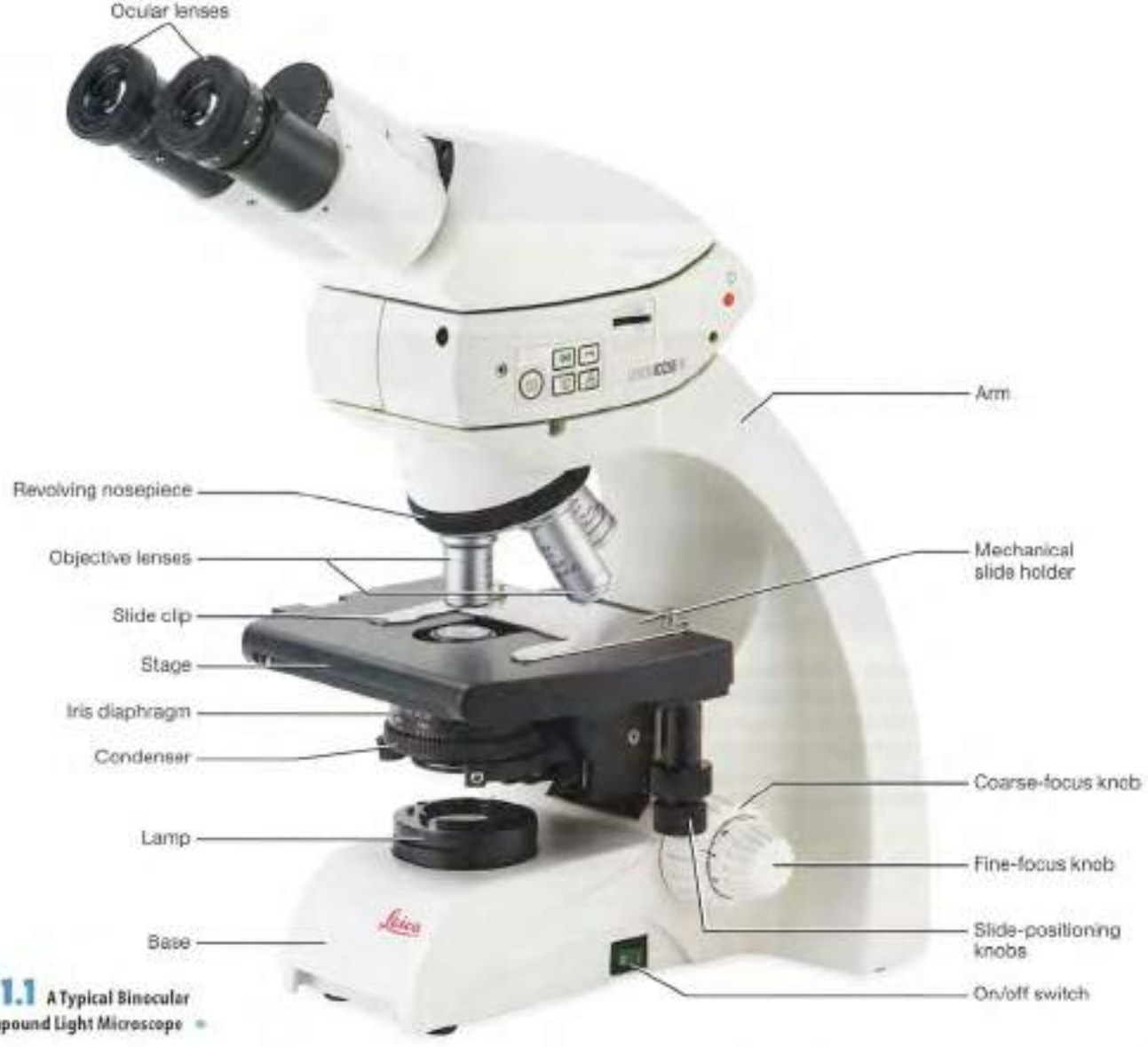


FIGURE **1.1** A Typical Binocular Compound Light Microscope

Compound light microscopes provide two levels of magnification, a design feature that *compounds* (increases) how enlarged the final image appears. The objective lens provides the first layer of magnification and the ocular lens provides the second. So, to determine the final image magnification, we multiply the magnification each of these lenses provides. Each lens has its magnification level marked on the lens barrel (Fig. 1.3).

$$\text{Final magnification} = (\text{Objective lens magnification}) \times (\text{Ocular lens magnification})$$

Most compound microscopes provide a maximum final magnification of 1000× (the "×" after this number indicates that this is a magnification level; you should apply this formatting when recording your magnification information for the lab exercise). Although higher magnifications are possible, increasing magnification beyond about 1300× yields a blurry image. This is because image **resolution** (image sharpness) tends to decrease as magnification increases. The **limit of resolution** (or **resolving power**) is a measurement of how far apart two points must be for us to see the points as separate (Fig. 1.4). Resolution *increases* (improves) as the limit of resolution *decreases*. Even the best light microscope will have a resolving power maximum of about 0.2 µm. That means even the best equipment can only allow us to see two points as distinct from one another if they are *at least* 0.2 µm apart.

FIGURE **1.2** **Three Types of Light Microscopy to View an Unstained Amoeba**

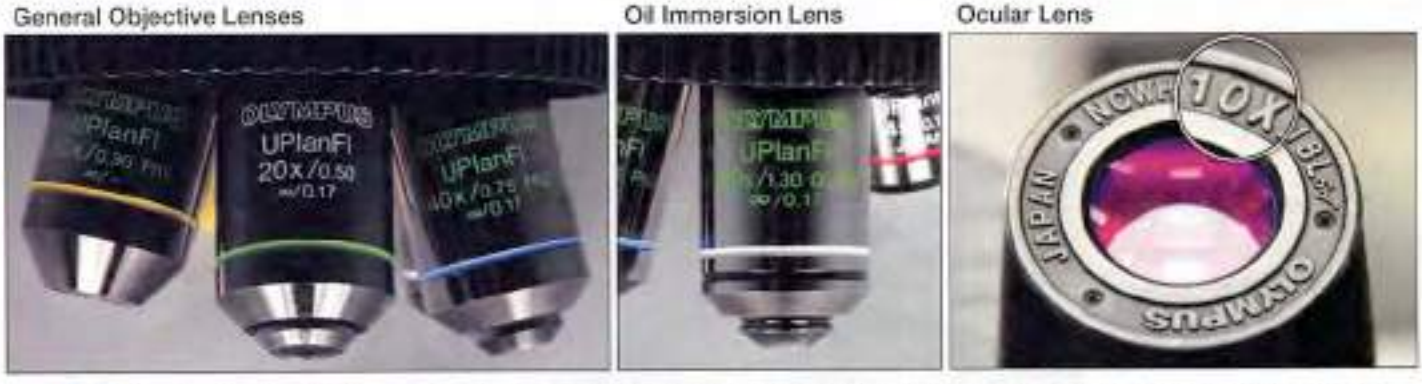


FIGURE **1.3** **Lens Magnification Markings** ● The markings on an objective lens barrel describe the lens' optical features. Here we focus on magnification markings. **Objective Lenses:** The yellow-banded lens provides a 10× magnification; the green-banded lens provides a 20× magnification; and the blue-banded lens provides a 40× magnification. These color bands are standard for quick magnification reference. Many microscopes also have a 4× lens, which would have a red band (not shown). **Oil-Immersion Lens:** This lens has two bands; the white band indicates the lens provides 100× magnification; and the black band indicates this lens must be used with immersion oil to obtain the sharpest image. Note that the word *oil* is also etched on the lens' barrel. Immersion oil can damage lenses that do not have an "oil" specification. **Ocular Lens:** The lens shown here provides a 10× magnification. Some ocular lenses are not 10×, so check the specifics before calculating final magnification.

FIGURE 1.4 Limit of Resolution • Most automobile headlights are about 1.5 m apart. Looking at the cars in the photo's foreground, it's easy to see a car's headlights as separate. The more distant cars appear smaller (but really aren't) as does the apparent distance between the headlights. When the apparent distance between the headlights reaches about 0.1 mm, they become indistinguishable as separate lights. This is because 0.1 mm is the human eye's limit of resolution.

Light's Journey through the Compound Light Microscope

The image we see in light microscopy results from how light interacts with the specimen and what light eventually makes its way to our eyes. Light's journey through the compound microscope starts at the **lamp** (or light source) located at the base of the microscope. From the lamp, light then passes through the **condenser**, a lens that focuses and aims the light onto the specimen. The condenser does not provide magnification, but it is important for improving image resolution.

The **iris diaphragm** is located just above the condenser. It allows you to control how much light illuminates the specimen; the more open the iris diaphragm, the more light aimed at the specimen. Microscopy may be many things, but it shouldn't be physically painful! If your eyes hurt because the view is too bright, you can reduce the brightness by adjusting the iris diaphragm so less light is shining onto the sample or you can dim the light using the **light-intensity knob**. Similarly, if you are straining to see the sample because it's too dark, open the iris diaphragm and/or increase the source lamp's intensity.

After light passes through the iris diaphragm, it passes through the glass slide and the sample mounted on the slide. Light that passes through the specimen and the glass slide then makes its way to the **objective lens**, the lens on the microscope's revolving nosepiece. This is the first lens that provides image magnification. From there, light makes its way to the **ocular lenses**, where the second and final image magnification occurs.

Oil Immersion in Bright-Field Microscopy

Most microbiology lab microscopes have a high-power oil-immersion lens. This lens has the word *oil* and 100× etched on the lens' barrel. To achieve the best image with such a lens, we must place oil on the slide and submerge the tip of the lens into the immersion oil.

But how can a tiny drop of oil make such a big difference in image resolution? The answer lies in the fact that light experiences **refraction** (bends) when it passes from one medium to a different medium, such as when it passes from water into air. Because air and water bend (refract) light differently, an underwater object appears blurry when you view it from above the water line.

By using immersion oil, we reduce the blurring that refraction produces. This is because immersion oil has a refractive index that is extremely close to that of glass. Therefore, by adding a drop of immersion oil to the surface of the slide and then submerging the tip of the oil lens into that oil, we effectively eliminate the air-glass interface that would otherwise have led to a blurry image.

Other Forms of Light Microscopy

Due to the light microscope's limited magnification and resolution potential, it's difficult to *directly* view things smaller than about 500 nm. This means light microscopy is not ideal to directly see microbes like *Mycoplasma*, a genera of bacteria that are about 150–350 nm in size and include pathogens such as *Mycoplasma pneumoniae* (the causative agent of walking pneumonia). Similarly, we cannot use light microscopy to directly view most viruses. That said, we can *indirectly* view *Mycoplasma* and viruses using a specialized form of light microscopy called **fluorescence microscopy** (Fig. 1.5). Although we rarely perform fluorescence microscopy in an introductory microbiology teaching lab, it's extremely useful in clinical microbiology labs.

As compared to fluorescence microscopy, which requires a highly specialized microscope, two other forms of light microscopy—**dark-field microscopy** and **phase-contrast microscopy**—are easily performed using a modified compound light microscope. Both phase-contrast and dark-field microscopy manipulate light waves and alter how light interacts with the specimen. They do so in such a way that they improve specimen contrast without needing to stain the specimen. Avoiding specimen staining can be desirable since most staining procedures distort and kill the specimen. For examples of dark-field and phase-contrast micrographs, refer to Figure 1.2.

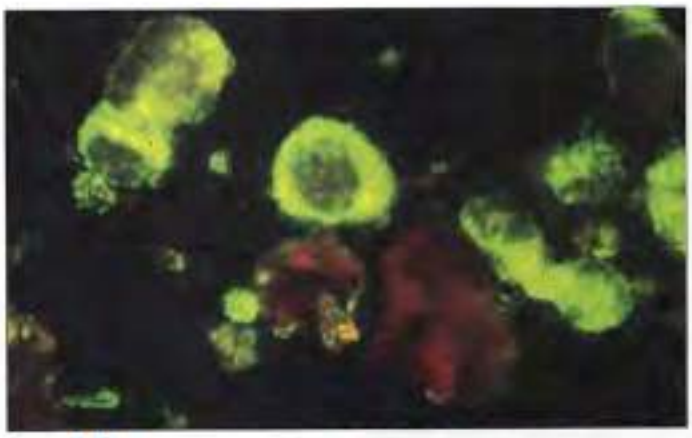


FIGURE **1.5** **Fluorescence Microscopy** ■ Fluorescence micrograph of *Mycobacterium kansasii*. The apple-green color is characteristic of fluorescence microscopy.

Clinical Applications

Light microscopes help health-care providers and lab personnel identify microbes in patient samples. As you may suspect, a compound microscope is indispensable in any microbiology lab, but did you know that many primary care providers and specialists (e.g., gynecology, dermatology, etc.) also have a compound light microscope available to help them perform more accurate point-of-care diagnosis? It's cheap, fast, and easy to use a compound light microscope to detect pinworm infections, urinary tract infections, trichomoniasis (a common sexually transmitted infection caused by the protozoan *Trichomonas vaginalis*), fungal skin infections, vaginal yeast infections, and bacterial vaginosis (vaginal inflammation caused by the overgrowth of certain bacteria that are normally present in the vagina).

When combined with staining techniques (which later lab activities cover), light microscopy is an indispensable diagnostic tool. In some cases, seeing the causative pathogen in a patient sample remains a "gold standard" for diagnosis. For example, seeing acid-fast bacteria in a patient's sputum (coughed-up mucus) is still a mainstay for diagnosing tuberculosis.

MATERIALS

- ❑ Compound light microscope
- ❑ Lens paper
- ❑ Lens-cleaning solution or 95% ethanol
- ❑ Immersion oil
- ❑ Letter "e" slide (Part A)
- ❑ Colored-threads slide (Part B)
- ❑ Prepared slides of bacteria and/or other microbes (instructor's choice) (Part C)

Using a Compound Light Microscope

These are generalized instructions that are useful for this lab and that you may also refer to whenever you are performing microscopy during the course. Your instructor may supplement the following with specifics related to your lab's equipment.

Getting the Microscope Ready for Use

1. If you must move the microscope, do so with both hands. Place one hand on the microscope's arm and the other under the base.
2. Carefully place the microscope on the table.
3. Unwind the power cord and plug in the microscope.
4. With gentle pressure, clean the microscope's ocular and objective lenses with lens paper and lens cleaner. Improper cleaning will scratch the lenses; follow your instructor's directions and preferences on this.
5. Turn on the lamp and adjust the lamp intensity to its highest level.
6. Slowly and gently turn the rotating nosepiece so that the scanning objective (4× objective lens) "clicks" into place over the slide. If your microscope lacks a 4× lens, position the low-power objective (10×) into place over the stage. Do not rotate the nosepiece by the lenses, this can damage the lenses and cause them to unscrew from the nosepiece.
7. Use the coarse-focus knob (the larger knob on the body of the microscope) to bring the stage to its highest position.

Focusing the Microscope Without Immersion Oil

1. Check that your microscope's stage is in the highest position and that the 4× objective is clicked into place over the stage.
2. Use the slide clip to secure the slide you want to view.
3. Center your specimen over the light. If your microscope has a mechanical slide holder, use the slide-positioning knobs to do this; otherwise, use your hand to gently position the slide.
4. With your eyes relaxed and slightly above the oculars, look into the microscope. If the light is too bright, dim the lamp and/or close the iris diaphragm. If using a binocular microscope, adjust the ocular lenses' spacing for your eyes so you see a single full circle of light.
5. While looking through the ocular lenses, slowly lower the stage by rotating the *coarse-focus* knob. Do this until you achieve the sharpest image possible. From this point on, you should no longer need to use the coarse-focus knob.
6. Now, while looking through the microscope, use the *fine-focus* knob to sharpen the image.
7. Scan the specimen and position it in the center of the field of view so you do not "lose" it upon advancing to a higher magnification.
8. Now, advance to a higher magnification by clicking the next objective into place over the specimen. Do not move your slide or the stage, just click the next objective lens into place. If you started with the 4× lens, you should now have the 10× objective in place.
9. Look into the microscope and, if necessary, slightly adjust the fine focus to sharpen the image. You can also adjust the image brightness if necessary. Some iris diaphragms have markings to show which setting works best for a specific objective lens. If your microscope has this feature, match the iris diaphragm setting with the objective lens you have in place.
10. Rotate the 40× objective into place and adjust the fine focus to sharpen the image.

Focusing the Microscope Using Immersion Oil

When viewing tiny cells such as bacteria or the details of a blood smear, the oil-immersion lens usually provides the best view.

1. Perform each step described in the "Focusing the Microscope Without Immersion Oil" section.
2. Once your specimen is in focus under the 40× objective, gently rotate the nosepiece to a position midway between the 40× lens and the oil-immersion lens (100× lens).
3. Do *not* move your stage down before adding the immersion oil or you will "lose" your focused image. Instead, as you "float" between the 40× and 100× objectives, place a drop of immersion oil onto the specimen. ***Do not drip oil on the microscope, especially its non-oil lenses. If you do, immediately clean off the oil with lens cleaner and lens paper.***
4. Now, slowly rotate the 100× lens so its tip is submerged in the oil drop, pass through it, and then return the lens into the oil. This minimizes the occurrence of air bubbles. ***Note:*** It may look as though the lens will not clear the slide. Provided you have followed all directions, it should click into place and the tip of the lens will be submerged in the oil. If the lens will not easily rotate into place, do not force it into place, you will break the slide and damage the oil lens. Instead, stop and ask for help.
5. ***Caution!*** This is another step where students tend to break slides. Use the fine-focus knob to *slowly* sharpen the image. If you encounter any resistance from the instrument's focus knob, stop and ask for help. Do not force a knob to turn; you will break the slide.
6. If the image appears dark, refer to Step 5 in the "Focusing the Microscope Without Immersion Oil" section.

Storing the Microscope

Follow these steps when you finish using your microscope.

1. Use the coarse-focus knob to completely lower the microscope's stage (or completely raise the objectives).
2. Remove the slide from the microscope's stage.
3. If you used immersion oil, use lens paper with lens cleaner to gently remove oil from any permanent slides you viewed before you return them to storage.
4. If you used immersion oil, use lens paper and lens cleaner to gently wipe any excess oil off the 100× lens.
5. "Click" the scanning objective (4× lens) into position over the stage.
6. Return the iris diaphragm to the fully opened state.
7. Turn off the lamp.
8. Unplug your microscope.
9. Wrap the electrical cord according to your instructor's preference.
10. If available, cover the microscope with a dust cover.
11. Return the microscope to its appropriate storage place.

PROCEDURE

Your instructor may have you do some or all parts of the following lab. Pay attention to their instructions and proceed in the order they direct you.

PART A: Viewing a Letter "e"

In this part of the lab, you'll view a macroscopic specimen (a letter "e"). This exercise helps to acquaint you with the microscope's parts and with focusing the microscope on a sample *without* using immersion oil. You'll also see that the images we observe are not always what they appear to be.

1. Follow each step in the "Getting the Microscope Ready for Use" section.
2. Obtain a letter "e" slide and examine it *without* the microscope. Record the orientation of the letter with the slide facing up and the label to the left. Sketch the orientation of the letter "e" on your data sheet.
3. Place the slide on the stage in the same position as you examined it with your naked eyes (label facing upward and positioned to your left).
4. Now, follow Steps 1–7 as described in the "Focusing the Microscope without Immersion Oil" section. For this exercise, you will only observe the letter "e" slide under the 4× objective.
5. Once you have the letter in focus, sketch the apparent orientation of the letter "e" on your data sheet. How, if at all, is the letter's orientation different from what you expected? Record your answer on your data sheet.
6. Now move the *slide* to the right. On your data sheet, record the direction the *image* moves.
7. Reposition the "e" in the center of the field. Move the *slide* toward you and on your data sheet, record the direction the *image* moves.
8. Remove the slide from the microscope and return it to its proper storage location.

PART B: Viewing Colored Threads

In this exercise, you'll view a macroscopic specimen (colored threads). This exercise provides additional practice with focusing the microscope on a sample *without* using immersion oil. You'll also experience the challenge of differentiating layers of detail in a specimen and you'll practice viewing a sample in different focal planes (positions where one layer of the sample appears in focus while the other layers appear blurry).

1. Obtain a colored-threads slide and view it with your naked eye (Fig. 1.6). Try to determine where in the stack each color resides. Is the red thread on the top, bottom, or middle? Do the same for the yellow and blue threads.
2. Now, follow Steps 1–7 as described in the "Focusing the Microscope without Immersion Oil" section.
3. Observe the slide with the 4× objective and try to determine the order of the threads. Record your observations on your data sheet. It may be helpful to observe the threads at various focal planes; you can use the fine-focus knob to do this.

4. Observe the slide with the 10× objective and try to determine the order of the threads. Record your observations on your data sheet. Observe the threads at various focal planes.
5. Now, click the 40× objective into place and observe the threads. Record what you see on the data sheet. Again, observe the threads at various focal planes.
6. Remove the slide from the microscope and return it to its proper storage location.

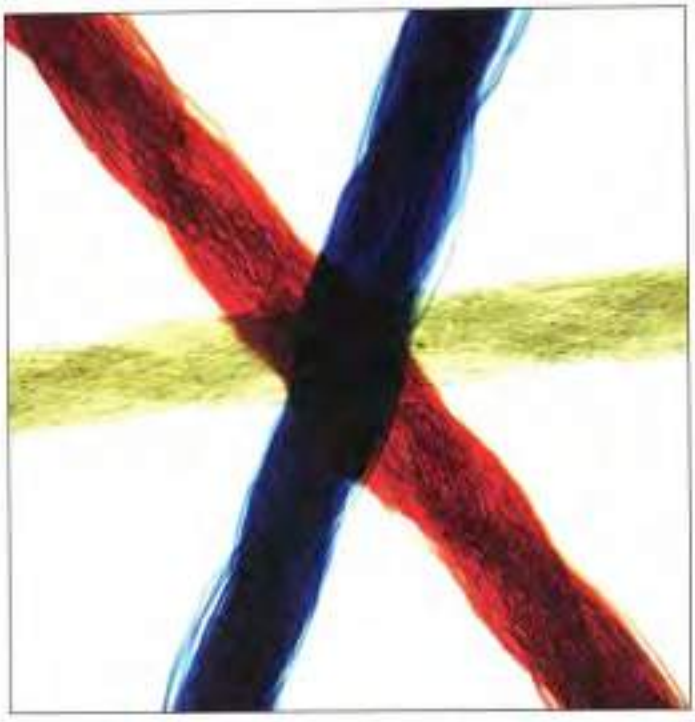


FIGURE **1.6** **Which Thread is on Top?** ● Even with the microscope, determining the order of threads from top to bottom is a challenge. This will require patience and use of the fine focus! Making it worse, not all the slides will be the same. Good luck!

PART C: Using Oil Immersion to View a Microscopic Specimen

In this exercise, you'll view at least one microscopic specimen selected by your instructor (e.g., bacteria, a blood smear, etc.). This exercise helps familiarize you with the parts of the microscope and with focusing the microscope on a sample using immersion oil.

1. Your instructor will tell you which slides to view for this exercise.
2. Clean the slide with lens paper and lens cleaner before you start.
3. Follow the steps as described in the "Focusing the Microscope Using Immersion Oil" section.
4. Document your observations on your data sheet.
5. When finished, follow the steps in the "Storing the Microscope" section.

1

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 1

DATA SHEET

Introduction to Light Microscopy

During the Lab

1 Based on the microscope you used, calculate the final magnification at which you viewed the letter "e" in Part A.

2 From Part A, sketch your letter "e" observations in the table provided. Be sure the slide is right-side up with the label oriented to your left.

Appearance of the "e" with the naked eye	While looking at the "e" under the microscope, how (if at all) is the letter's orientation different from what you expected?	When the stage moves to the right, the image moves...	When the stage moves toward you, the image moves...

3 Record your observations of the colored threads for each scenario in the table provided.

Naked eye	Top:	Middle:	Bottom:
4× Objective lens	Top:	Middle:	Bottom:
10× Objective lens	Top:	Middle:	Bottom:
40× Objective lens	Top:	Middle:	Bottom:

4 In the sketch circle provided, draw your observations for *at least* one slide that you viewed with oil immersion. (Your instructor will designate what slides you should view.)

Specimen Label: _____

Image's final magnification (show your calculation below): _____

Lab Related Questions

1 Color the microscope parts as indicated.

Coarse-focus knob (orange)

Fine-focus knob (purple)

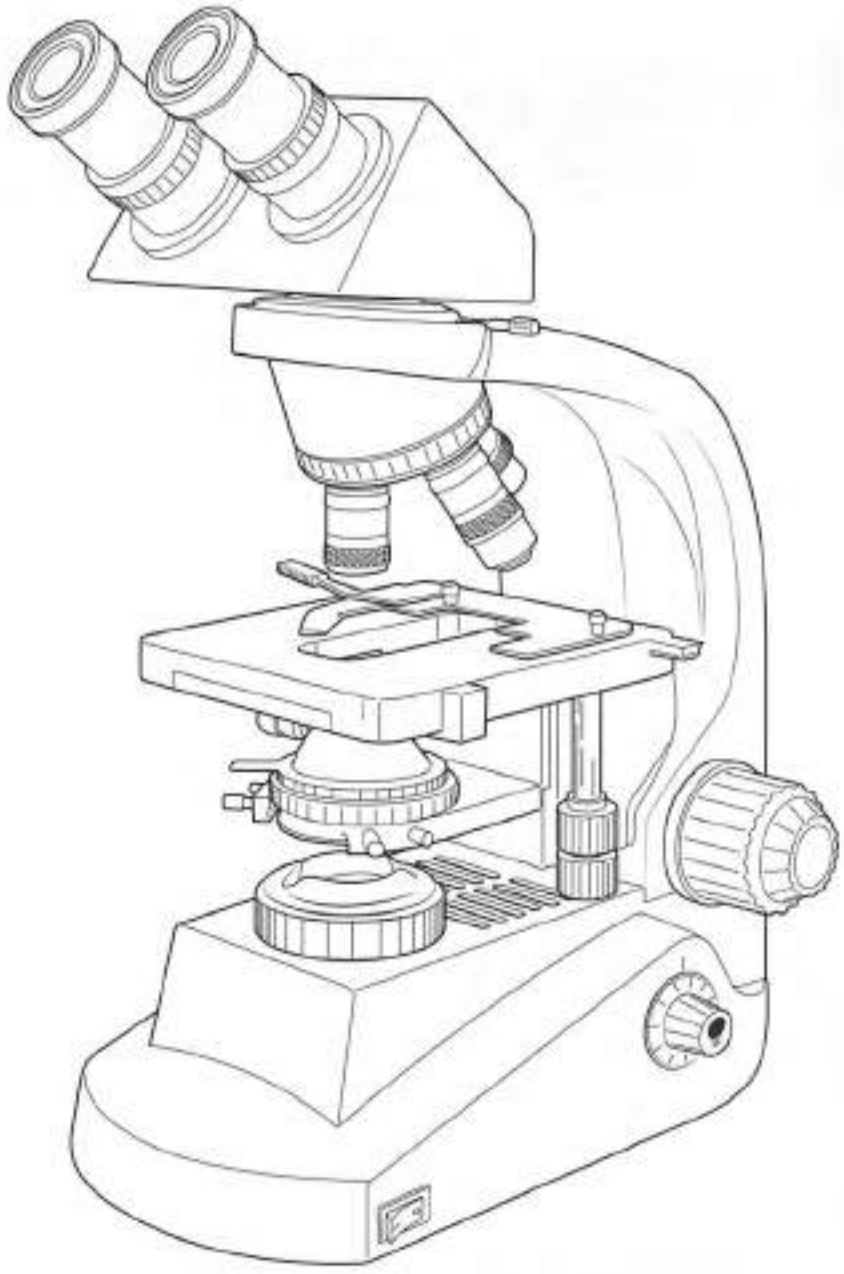
Slide-positioning knobs (yellow)

Objective lenses (red)

Ocular lenses (blue)

Revolving nosepiece (black)

Slide clip (green)



Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 1
DATA SHEET
(continued)

2 Assume you view a specimen at a final magnification of 55×. If the objective lens was 5×, then what was the microscope's ocular lens magnification rating?

a. 5×
b. 10×
c. 11×
d. 55×
e. Not enough information

3 Which of the following could help you detect a virus in a patient sputum sample?

a. Bright-field microscopy
b. Dark-field microscopy
c. Phase-contrast microscopy
d. Fluorescence microscopy
e. None of the above

4 Some steps listed should *not* be used if you want to successfully view a specimen via oil immersion. First, draw a line through the steps you would NOT use. Next, place the remaining steps in sequential order, with "1" being the earliest step.

_____ Place oil on the slide.

_____ Use the coarse-focus knob to focus while viewing the sample with the 100× lens.

_____ Use the fine-focus knob to focus the specimen under 10× magnification.

_____ "Float" between the 40× and 100× objective lenses.

_____ Focus the specimen under 40× magnification.

_____ Lower the stage so you may more easily apply oil onto the slide.

_____ Place your slide on the stage.

_____ Rotate the 100× lens into place.

5 As magnification increases, _____ tends to decrease.

6 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ Light microscopy can help us directly view viruses.

_____ A microscope with a resolving power of 0.2 µm would be described as having a lower resolving power than a microscope with resolving power of 0.3 µm.

_____ Immersion oil improves image resolution by reducing light refraction.

_____ The 40× objective lens is also known as the scanning objective.

_____ The final magnification of an image is determined by multiplying the level of magnification provided by the ocular lens by the magnification provided by the objective lens.

7 Name the microscope part:

_____ Opens and closes to modulate how much light is aimed at the specimen.

_____ The part of the microscope that focuses the light aimed at the specimen.

_____ The only objective lens that should encounter immersion oil.

_____ The lens that provides the second and final image magnification.

8 Resolving power is best described as the ability to _____.

a. keep two points separated
b. differentiate between two distinct points
c. merge two distinct points
d. magnify a specimen
e. refract light

Critical Thinking

1 Why is wavelength the main limiting factor for resolution in light microscopy?

2 Would you expect water to work as well as oil for oil-immersion microscopy? Explain your reasoning—*be specific.*

3 If you do not adjust the lamp intensity or the iris diaphragm, you will notice that your specimen appears darker when viewed via the 100× lens as compared to its appearance when viewed via the 40× lens. Explain why this normal optical effect occurs.

EXERCISE 2

Microscopic Examination of Eukaryotic Microbes

After doing this exercise, you should be able to:

1. Describe the basic differences between prokaryotes and eukaryotes.
2. Name the three domains and six kingdoms of taxonomy.
3. List the taxonomic hierarchy categories from broadest to most specific.
4. Correctly write a scientific name.
5. List several features of kingdom Fungi and name some representative members.
6. List several features of kingdom Protista and name some representative members.
7. Define the term *protozoan* and name the four main groups of protozoans.
8. State what feature is primarily used to group protozoans into their four main categories.
9. Describe the features of select fungi viewed on premade and/or wet-mount slides.
10. Describe the features of select protists viewed on premade and/or wet-mount slides.

Background

Cells are described as being either prokaryotic or eukaryotic. **Prokaryotic** cells are unicellular (single-celled) microbes that have ribosomes, but lack a nucleus and other membrane-bound organelles (e.g., mitochondria, Golgi apparatus, endoplasmic reticulum, etc.). In general, prokaryotes are smaller and genetically simpler than their eukaryotic cousins (Fig. 2.1). In contrast, **eukaryotic** cells have a nucleus along with other membrane-bound organelles. Among the eukaryotes, we find unicellular and multicellular organisms. Although most of this book focuses on prokaryotes, here we'll focus on the eukaryotes with a specific emphasis on fungi and protists.

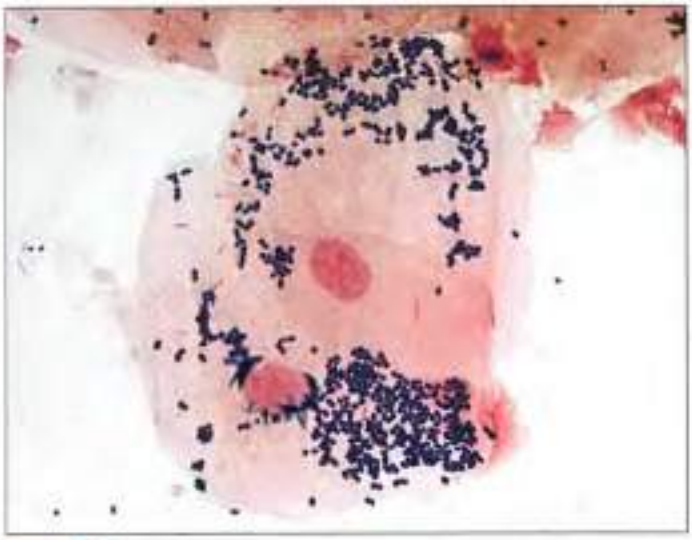


FIGURE 2.1 Bacteria and Eukaryotic Cells • This Gram-stained specimen was taken from just below the gum line in the mouth. The large, pink cells are human epithelial cells; notice the prominent nucleus in each cell. The small, purple and pink cells are bacteria, which are typically smaller than eukaryotic cells, roughly 1 µm to 5 µm in length versus 10 µm to 100 µm for eukaryotic cells.

Taxonomy and Eukaryotic Organisms

Taxonomy is a cataloging system that arranges living things into groups. The broadest taxonomic category is called a **domain** while the most specific is the **species** level. There are currently three taxonomic domains: Archaea, Bacteria, and Eukarya. Prokaryotes fall within the first two domains (Archaea and Bacteria) while eukaryotes are grouped within domain Eukarya. Based on genetic evidence, domain Eukarya shares a common evolutionary ancestor with the prokaryotes (Fig. 2.2).

Classifying organisms beyond the domain level has been a taxonomic battleground. The latest development in taxonomy was to divide domain Eukarya into seven **superkingdoms** (or supergroups). This newer classification model eliminates the more widely known kingdom model (Fig. 2.3).

While the superkingdom model more accurately reflects how eukaryotic organisms are genetically related, it adds complexity to learning the basic observable features of microorganisms. Because our main goal is to explore basic microbial features as opposed to genetics, in this lab we'll use the older kingdom model.

The number of kingdoms in the taxonomic hierarchy has varied over the years with the total number ranging from five to eight. For our purposes, we'll apply the six-kingdom model. In this model, prokaryotes are spread across **kingdom Bacteria** and **kingdom Archaea** while eukaryotes span four kingdoms: **Animalia**, **Plantae**, **Fungi**, and **Protista**. Regardless of how many kingdoms we acknowledge, each is further subdivided into more precise groupings as follows: phylum, class, order, family, genus, and species, with species being the most specific grouping.

An organism's scientific name includes its genus and species designation. All scientific names are written with the genus name capitalized and the species name lowercased. The entire scientific name is then either underlined or italicized. When writing a scientific name, you should first write the full name. In later references, you can abbreviate the name by writing the first letter of the genus as an uppercase letter followed by a period and the full species name in lowercase letters. As with a full scientific name, abbreviated names should be either underlined or italicized (Fig 2.4).

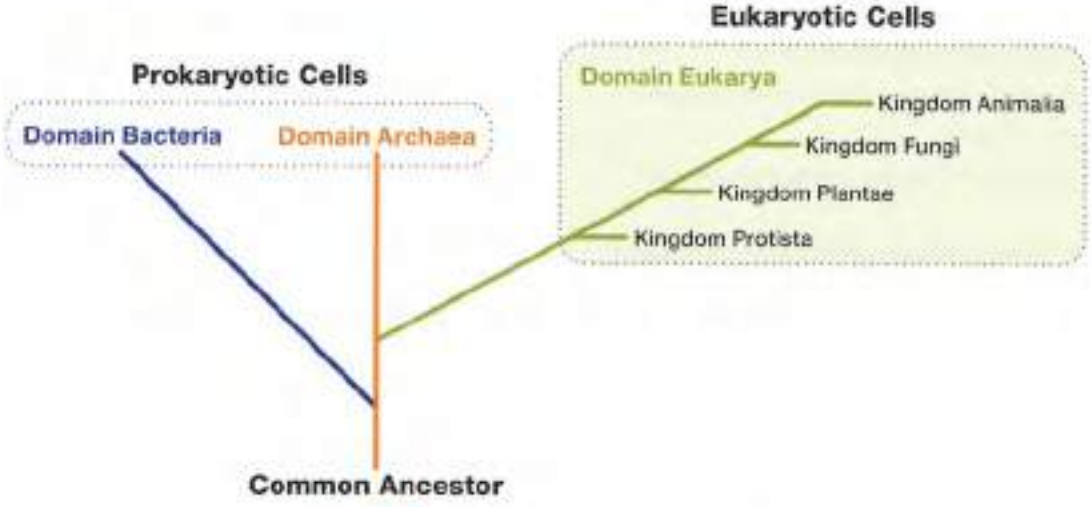


FIGURE **2.2** **The Three Taxonomic Domains of Life** •

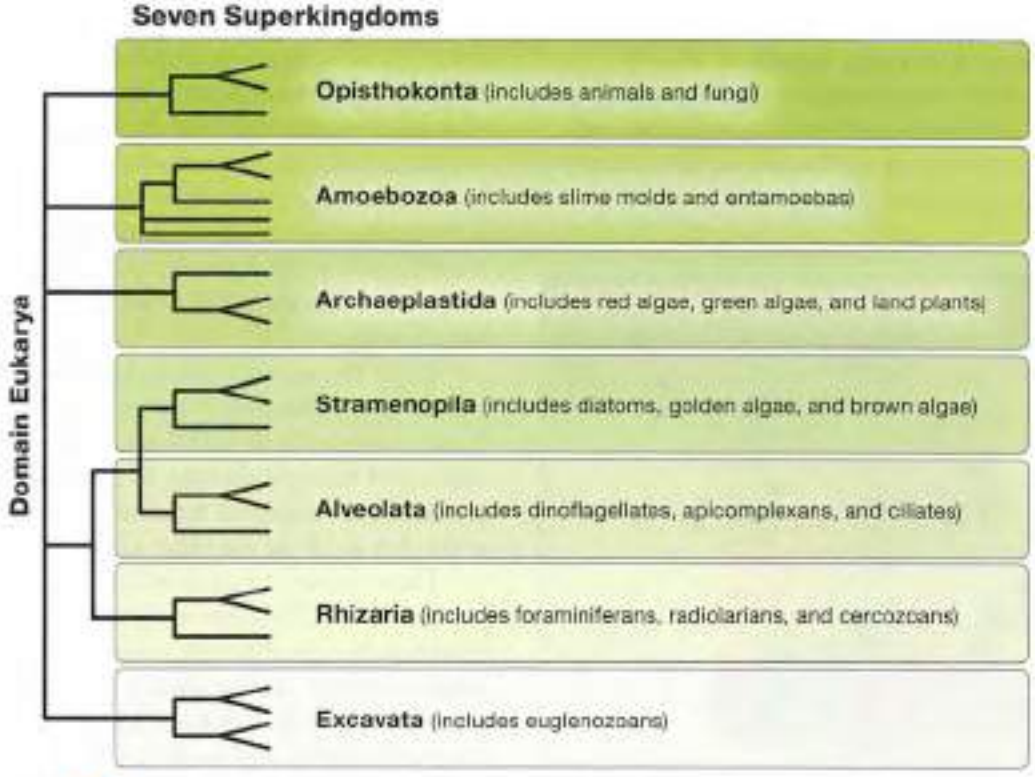

Seven Superkingdoms
Domain Eukarya
Opisthokonta (includes animals and fungi)
Amoebozoa (includes slime molds and entamoebas)
Archaeplastida (includes red algae, green algae, and land plants)
Stramenopila (includes diatoms, golden algae, and brown algae)
Alveolata (includes dinoflagellates, apicomplexans, and ciliates)
Rhizaria (includes foraminiferans, radiolarians, and cercozoans)
Excavata (includes euglenozoans)

FIGURE **2.3** **Domain Eukarya's Seven Superkingdoms** • Genetic analyses have helped scientists organize domain Eukarya into superkingdoms. The superkingdom model's details are still being debated. When organisms are grouped based on their genetics, it's easier for us to see that diatoms are closely related to certain algae while fungi are more closely related to animals than to slime molds.

Broadest category

Most specific category

Example	
Domain	Eukarya
Kingdom	Fungi
Phylum	Ascomycota
Class	Eurotiomycetes
Order	Eurotiales
Family	Trichocomaceae
Genus	Penicillium
Species	Chrysogenum
Full scientific name: *Penicillium chrysogenum* Abbreviation: *P. chrysogenum*	

FIGURE **2.4** **Taxonomic Hierarchy and Scientific Names** • You can remember the taxonomic hierarchy order with the following mnemonic device: Dear King Philip came over for great spaghetti.

Exploring Kingdom Fungi

Fungi (singular: fungus) are nonmotile, non-photosynthetic eukaryotes. Their cell wall is usually made of the polysaccharide **chitin**, not cellulose as in plants. Aside from pathogenic fungi, most fungi are **saprobes**, meaning they get their nutrients from decomposing organic matter. Based on their appearance, fungi are informally divided into **filamentous molds** and **unicellular yeasts**.

Molds

Molds grow as filaments of cells. These filaments are called **hyphae** (singular: hypha) and collectively form a **mycelium**. Molds produce a variety of sexual and asexual reproductive spores, which are used as a basis for classification. Three genera of molds will be covered here: *Rhizopus*, *Aspergillus*, and *Penicillium*.

- *Rhizopus*

  The dozen or so *Rhizopus* species are fast-growing molds that produce white or grayish, cottony growth. The mycelium becomes darker with age as sporangia form, giving it a "salt and pepper" appearance (Fig. 2.5A). *Rhizopus* species produce broad surface and aerial hyphae with irregular diameters. Anchoring **rhizoids** form where the surface hyphae (**stolons**) join the bases of the long, unbranched **sporangiophores** (Fig. 2.5B).

  Rhizopus's life cycle has both sexual and asexual phases. Asexual **sporangiospores** are produced by large, circular sporangia borne at the ends of the elevated sporangiophores (Fig. 2.5C). A hemispherical **columella** supports the sporangium. After release, the sporangiospores develop into hyphae identical to those that produced them.

  On occasion, sexual reproduction occurs when hyphae of different mating types (designated + and – strains) make contact. Initially, **progametangia** extend from each hypha and nuclei and cytoplasm flow into them (Fig. 2.5D). As a consequence, they enlarge and contact one another. Upon contact, a septum separates the end of each progametangium into a **gametangium** and a **suspensor** (Fig. 2.5E).

  The walls between the two gametangia dissolve, and a thick-walled **zygosporangium** develops (Fig. 2.5F). Fusion of nuclei occurs within the zygosporangium, resulting in multiple diploid zygote nuclei. After a dormant period, zygote meiosis occurs and produces haploid **zygospores** which, when released, develop into new hyphae and the life cycle is completed (Fig. 2.5G).

  Two *Rhizopus* species (*R. oryzae* and *R. microsporus*) are primarily responsible for producing **zygomycosis** (also called mucormycosis). This infection is found most frequently in diabetics, organ transplant recipients, and other immunocompromised patients. Portal of entry and overall patient health are critical factors in determining the location and extent of infection. If untreated, the mortality rate is high, between 47–84% worldwide. On a less severe note, most species are harmless saprophytes. *R. stolonifer* is the common bread mold.

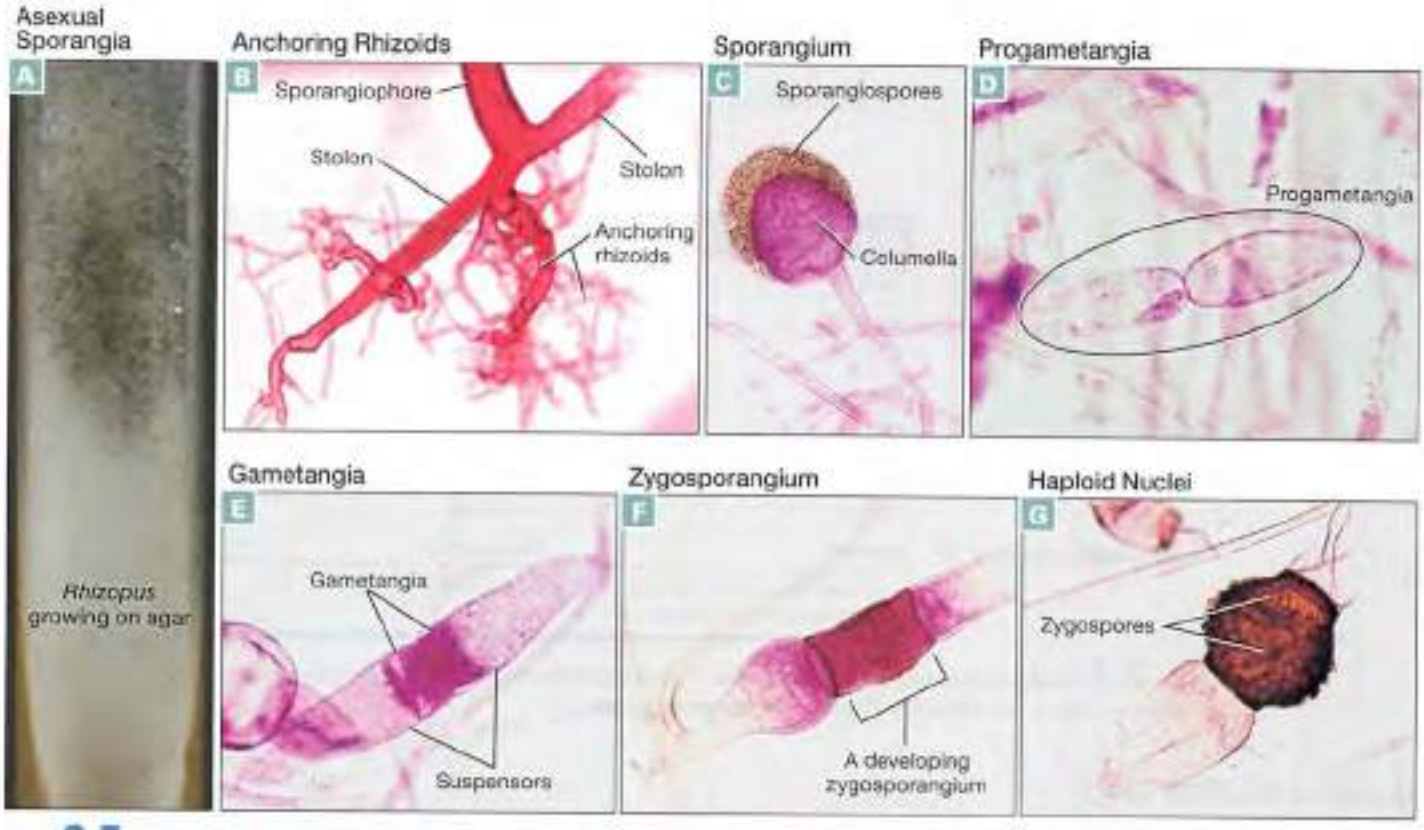


FIGURE 2.5 Mold *Rhizopus* • (A) **Asexual Sporangia:** The black asexual sporangia of the bread mold Rhizopus stolonifer give the mold a "salt and pepper" appearance. (B) **Anchoring Rhizoids:** Anchoring rhizoids form at the junction of each sporangiophore and the stolon. Note the absence of the septa—divisions between cells. (C) **Sporangium:** The sporangium is found at the end of a long, unbranched, and aseptate sporangiophore. The haploid asexual sporangiospores in the sporangium cover the surface of the columella, which has a flattened base. (D) **Progametangia:** The circled progametangia developed from different hyphae and will eventually fuse to form a gamete. (E) **Gametangia:** Gametangia and suspensors are shown. Gametangia contain one or more haploid nuclei from each mating type. (F) **Zygosporangium:** A zygosporangium forms when the cytoplasm from the two mating strains fuse. (G) **Haploid Nuclei:** Haploid nuclei from each strain fuse within the zygosporangium to produce many diploid zygote nuclei. (Commercially prepared slides.)

- ***Aspergillus***

Aspergillus species are commonly found in soil and on plants. Roughly 180 species have been identified. They form green to yellow or brown granular colonies with a white edge. One species, *A. niger*, produces distinctive black colonies. The *Aspergillus* "fruiting body" is distinctive, with chains of spores (called conidia) arising from one or two rows of cells attached to a swollen **vesicle** at the end of an unbranched stalk (Fig. 2.6A). Fruiting body structure and size, and conidia color are useful in species identification. It used to be thought that *Aspergillus* species were unable to sexually reproduce, but evidence is mounting that under the right environmental conditions sexual spores, called **ascospores**, are made.

A. fumigatus and other species are opportunistic pathogens that cause aspergillosis, an umbrella term covering many diseases. Immunocompromised patients have the highest risk of infection. Allergic aspergillosis may occur in individuals who are in frequent contact with the spores and become sensitized to them. Subsequent contact produces symptoms similar to asthma. Aspergilloma (fungus ball) involves colonization of the paranasal sinuses or lungs, resulting in abscess formation. It is frequently asymptomatic and resolves without treatment, but in serious cases it can be fatal if not treated.

Some *Aspergillus* species are commercially important. *A. oryzae* is used in the production of sake (made from rice), and soy paste (made from soybeans), which may be further fermented with *A. oryzae* and *A. soyae* into soy sauce. Aspergilli are also used in commercial production of citric acid, various enzymes, and many other products. Further, *A. niger* and *A. oryzae* have been designated as GRAS (generally regarded as safe) by the Food and Drug Administration and World Health Organization, which has opened the door for using them as hosts in biotechnological applications.

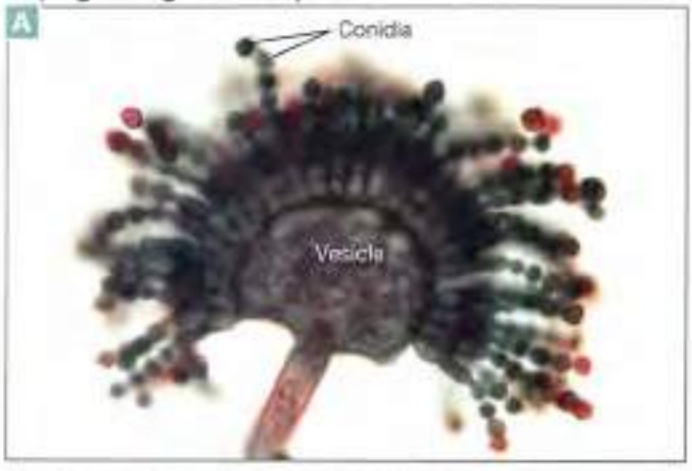
- ***Penicillium***

Members of the genus *Penicillium* are everywhere, being found in the air, soil, and decaying organic matter worldwide. Over 200 species have been

identified. They produce distinctive green, powdery, radially furrowed colonies with a white edge. *Penicillium* fruiting bodies are distinctive, looking like a brush (penicillus means "paintbrush") at the end of an elevated stalk (Fig. 2.6B).

Penicillium is best known for making the antibiotic penicillin (discovered by Alexander Fleming in 1928), but it is also a known **opportunistic pathogen** (an agent that mainly afflicts a weakened host). For example, *P. marneffei*, is endemic to Asia and is responsible for opportunistic infections of the lungs, liver, and skin in immunosuppressed and immunocompromised patients. *P. chrysogenum* is common in damp, indoor habitats and their spores can cause allergic reactions. Other species are important because they cause fruit and vegetable spoilage. Some *Penicillium* species are commercially important for making cheeses, *P. roquefortii* helps make Roquefort cheese and *P. camembertii* is used to make Camembert and Brie cheeses.

Aspergillus niger Conidiophore



Penicillium's "Brush-like" Conidiophore

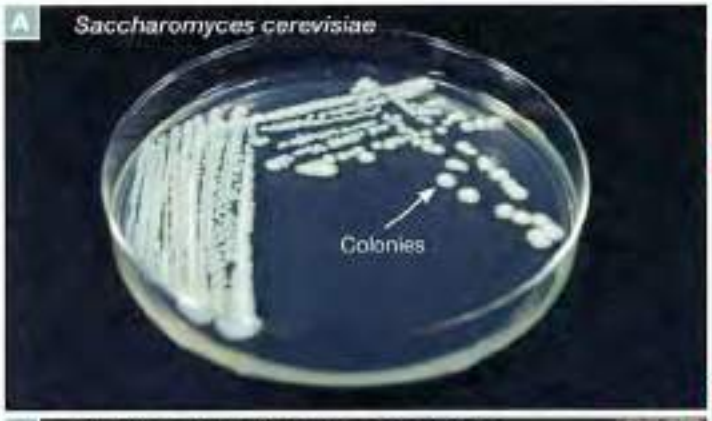
FIGURE **2.6** **Examples of Conidiophores: Molds *Aspergillus* and *Penicillium*** • (A) ***Aspergillus:*** Note the chains of spores (conidia) extending from the surface of the vesicle. (B) ***Penicillium:*** *Penicillium* species produce a characteristic brush-shaped fruiting body with chains of conidia. (Commercially prepared slides.)

Yeasts

Yeasts are the only unicellular fungi, but they can arrange themselves into short chains of cells. Two types of yeast are covered here: *Saccharomyces cerevisiae* and *Candida albicans*.

- ***Saccharomyces cerevisiae***
  Saccharomyces cerevisiae is used to make bread, wine, and beer but it is not an important human pathogen. It does not form a mycelium but, rather, produces a colony similar to bacteria (Fig. 2.7A). Vegetative cells are generally oval to round in shape, and asexual reproduction occurs by budding (Fig. 2.7B). Short **pseudohyphae** are sometimes produced when the budding cells fail to separate. In an *S. cerevisiae* population, haploid and diploid cells are indistinguishable from one another and both reproduce asexually by budding. Diploid cells occasionally undergo meiosis and produce one to four haploid **ascospores**. When released, ascospores bud to produce more haploid cells. Occasionally, haploid cells of opposite mating types combine to create a new diploid cell, thus acting like gametes.
- ***Candida albicans***
  Candida albicans is part of the normal upper respiratory, skin, gastrointestinal, and urogenital microbiota of men and women. It's also the most common fungal opportunistic pathogen (Fig. 2.7C). It is the causative agent of thrush in the oral cavity, vulvovaginitis of the female genitalia, and cutaneous candidiasis of the skin. Individuals most susceptible to *Candida* infections are diabetics, those with immunodeficiency (e.g., AIDS), catheterized patients, and individuals taking antimicrobial medications that disrupt the normal microbiota balance. Despite common belief, men can develop a yeast infection of the penis, although yeast infections are far more common in women.

  Candida albicans are round to oval cells that reproduce by budding and are 3–6 μm in diameter. Sometimes the cells separate after budding, but other times they remain attached, forming branched chains of two types: **pseudohyphae**, which are narrower at their junctions, and true hyphae which are not. Both types are asexual spores. The ability to switch between pseudohyphae and true hyphae may increase virulence. The sexual life cycle is similar to *S. cerevisiae*.



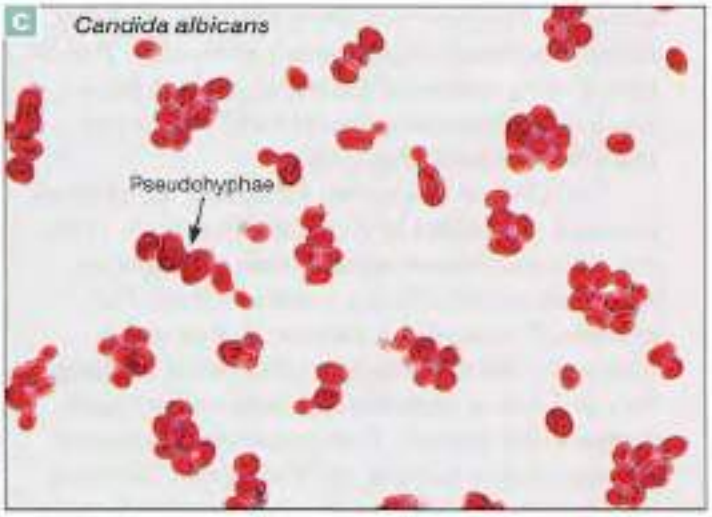


FIGURE **2.7** **Yeasts *Saccharomyces* and *Candida*** • (A) ***Saccharomyces cerevisiae:*** The brewer's yeast *Saccharomyces cerevisiae* colonies resemble bacterial colonies. Mold colonies present a more "fuzzy" appearance. (B) **Wet Mount *Saccharomyces cerevisiae:*** *S. cerevisiae* is shown in a wet-mount preparation using phase contrast microscopy. The cells are oval with dimensions of 3–8 µm by 5–10 µm. (C) ***Candida albicans:*** *Candida albicans* cells grown on a solid medium don't show natural arrangements seen in tissue samples. However, in this micrograph the oval cell shape and a pseudohyphae with a budding cell at its end are visible.

Exploring Kingdom Protista

In the six-kingdom model, kingdom Protista is a miscellaneous category encompassing organisms not assigned to the other three eukaryotic kingdoms: Animalia, Plantae, or Fungi. Protists can be described as plant-like, fungi-like, and animal-like. Some protists are photosynthetic, while others are not.

Plant-like Protists

Algae are examples of plant-like protists. These organisms are photosynthetic, typically have cellulose cell walls, and they often grow in fresh water. Most algae grow as unicellular, filamentous, or colonial species. Some unicellular algae, such as *Chlamydomonas*, use flagella for motility (Fig. 2.8A). *Volvox* is an example of a colonial species of green algae (Fig. 2.8B). *Volvox* colonies grow as spherical aggregates that contain about 50,000 cells. These algae can undergo sexual and asexual reproduction. *Spirogyra* is an example of a filamentous algae, it grows as long strands, which is why it's sometimes commonly known as "mermaid's tresses" or "water silk" (Fig. 2.9).

Diatoms are another example of a plant-like protist. These organisms, which are often referred to as microalgae, can exists as single-celled or colonial photosynthetic autotrophs (Fig. 2.10). They have a distinctive golden-brown color due to the abundance of the pigment

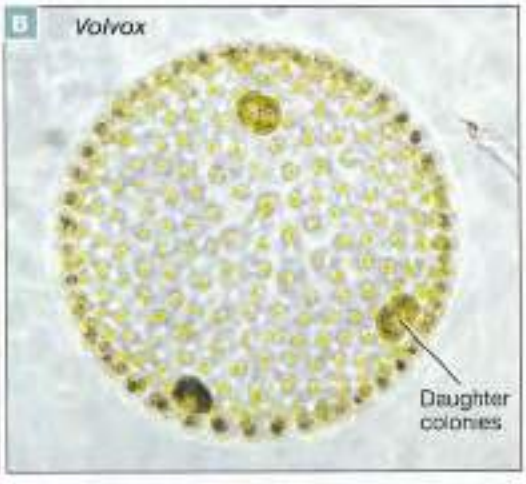


FIGURE **2.8** **Plant-like Protists: Green Algae** • (A) ***Chlamydomonas:*** *Chlamydomonas* is a unicellular green alga with a single, cup-shaped chloroplast. A red-orange stigma is found within the chloroplast and is involved in phototaxis (attraction to light). It also has two flagella for locomotion. Cells are approximately 30 µm long and 20 µm wide. (Wet mount of an environmental sample.) (B) ***Volvox:*** *Volvox* is a spherical colonial green alga made of cells similar in shape to *Chlamydomonas*. Daughter colonies form asexually from special cells in the parent colony. Release of a daughter colony involves its eversion as it exits through an enzymatically produced pore. Mature colonies can reach a size visible to the naked eye. (Wet mounts from culture.)

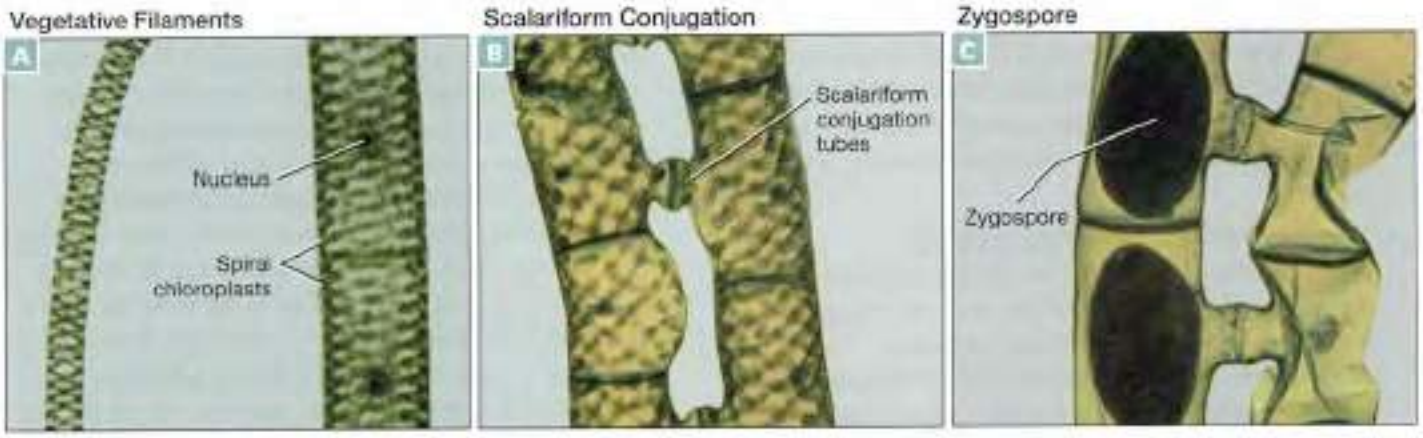


FIGURE 2.9 Plant-like Protists: *Spirogyra* ● *Spirogyra* is a filamentous algae with distinctive spiral chloroplasts. The cells are haploid, that is, they have only one complete set of chromosomes. (A) **Vegetative Filaments:** Two vegetative filaments are shown here. Also note the nuclei in the cells on the right. (B) **Scalariform Conjugation:** These two filaments have begun scalariform conjugation, a sexual process that occurs between different filaments. The protrusions are called conjugation tubes and serve as a connection through which the cytoplasm of one cell (acting as a gamete) can move and join with the cytoplasm of a cell in the other filament. (C) **Zygospore:** The cells and their two haploid nuclei fuse to form a diploid zygospore and conjugation is complete. When conditions are favorable, the zygospore germinates by undergoing meiosis. Only one haploid nucleus survives and the zygospore cell develops into a new vegetative filament. Note the absence of spiral chloroplasts in the empty cells and in the zygospores. (Commercially prepared slides.)

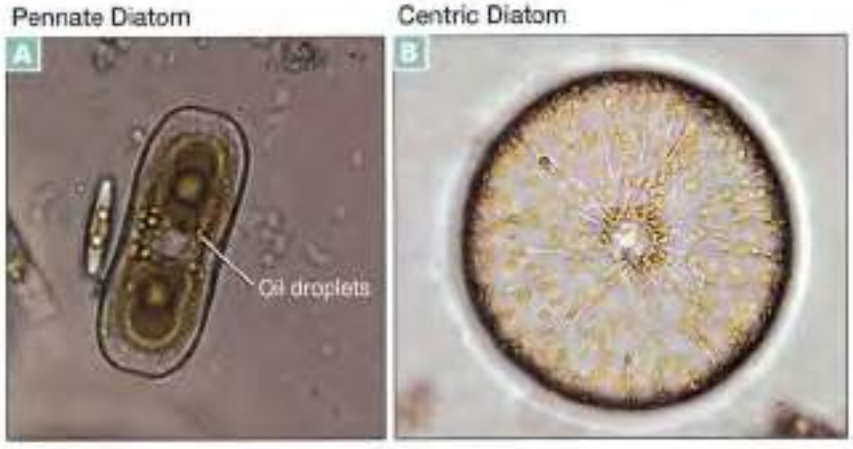


FIGURE 2.10 Plant-like Protists: Diatoms ● Diatoms are composed of silica and organic material. They have a cell wall that is divided into two halves, one of which fits into the other like the lid and base of a petri dish, and they are divided into two groups based on their symmetry. (A) **Bilateral symmetry:** Diatoms with bilateral symmetry are described as pennate. (B) **Radial Symmetry:** Diatoms with radial symmetry are described as centric. Notice the distinctive golden chloroplasts, the cell wall ornamentation, and the oil droplets. (Wet-mount preparations of environmental samples.)

fucoxanthin. Their highly ornamented cell wall is made of silica embedded in an organic matrix and consists of two halves with one half overlapping the other in the same way the lid of a petri dish overlaps its base. **Centric diatoms** have a round shape, whereas **pennate diatoms** are bilaterally symmetrical. Oil droplets are frequently visible inside diatoms. None of the plant-like protists are known to cause diseases in humans or other animals.

Fungi-like Protists

Some protists have more in common with fungi than they do plants or animals. These fungi-like protists are non-photosynthetic and instead live as saprobes. An example includes **slime molds**. Slime molds are unicellular organisms, but they can aggregate into a multicellular state to reproduce. If you've ever looked at a decaying tree on the forest floor or a wood-mulched flower bed and seen a yellow blob that looks like yellow dog-vomit, you've likely seen a slime mold. None of the fungi-like protists are known to cause diseases in humans or other animals.

Animal-like Protists

The animal-like protists can be free living or parasitic. They include a wide range of pathogens that afflict humans and other animals. Sometimes called **protozoans** (or protozoa) these organisms are unicellular, lack a true cell wall, exhibit asexual and sexual reproduction, and are typically non-photosynthetic, although some, like *Euglena*, are photosynthetic. Protozoans are organized into four groups based on their means of motility in their adult form.

- **Amoeboid Protozoans (Sarcodina)**
  All amoebas move by forming cytoplasmic extensions called **pseudopods**, which are also used to engulf their food. Sarcodines reproduce sexually by syngamy (fusion of two gametes) and asexually by division

or budding, they do not have a cell wall, and they can be free-living or parasitic. Classical amoebas are free-living in marine, freshwater, and soil environments (Fig. 2.11A). Another group of amoebas are the **entamoebas,** some of which are parasitic (Fig. 2.11B).

- **Flagellated Protozoans (Mastigophora)**
These protozoans include free-living microbes like *Euglena* and parasitic microbes such as *Trypanosoma* species, and *Trichomonas vaginalis*.

Euglena live as photosynthetic autotrophs when light is available and when light is not available, they can live as heterotrophs (ingest and breakdown an external organic carbon source for energy). They usually have two flagella, or more rarely only one. The red "eyespot" is a distinctive characteristic that is easily seen with the light microscope (Fig. 2.12A).

Trypanosoma species are heterotrophic and include important human pathogens. Two species (*T. brucei gambiense* and *T. brucei rhodesiense*) are pathogens that cause West African trypanosomiasis and East African trypanosomiasis, respectively (Fig. 2.12B). The former is generally a mild, chronic disease that may last for years before the nervous system is affected, whereas the latter is more acute.

Trypanosomes have a complex life cycle involving the tsetse fly (genus *Glossina*) as an intermediate host, which transmits the pathogen to the human host during a blood meal. The symptoms of sleeping sickness—sleepiness, emaciation, and unconsciousness—begin when the central nervous system becomes infected. Depending on the infecting strain, the disease may last for months or years, but without drug treatment the mortality rate is high.

Trichomonas vaginalis is the causative agent of trichomoniasis in humans (Fig. 2.12C). It is the most common pathogenic protozoan infection in people who live in industrialized nations. Indeed, it is the most common sexually transmitted eukaryotic infection. The parasite can cause inflammation of genitourinary mucosal surfaces. About 70% of cases are asymptomatic, but when symptoms do develop patients may experience itching in the penis or vagina, burning upon urination, and a discharge from the penis or vagina. An infection during pregnancy has been linked to preterm labor and lower birth weight babies. Trichomoniasis can be effectively treated with antiparasitic drugs.

- **Ciliated Protozoans (Ciliophora)**
Ciliates have hair-like appendages called **cilia** covering their outer surface. Cilia provide motility when they sweep back-and-forth. And because they line the **oral groove,** they also sweep food particles inward toward the **cytostome** for ingestion. Ciliates are common in aquatic environments and include diverse species from free-living *Paramecium* species and *Stentor* to parasites like *Balantidium coli*, the only ciliated protozoan known to cause human disease (Fig. 2.13).

Paramecium and *Stentor* are non-pathogenic ciliates that feed on organic matter in their environments. *Paramecium* species are found in marine and freshwater environments and are active swimmers as they search for food. *Stentor* species are found in freshwater and, although they can swim, they spend most of their time anchored to a surface as they feed.

Balantidium coli causes a rare form of dysentery called balantidiasis. This disease is found worldwide, but it's most prevalent in tropical and subtropical

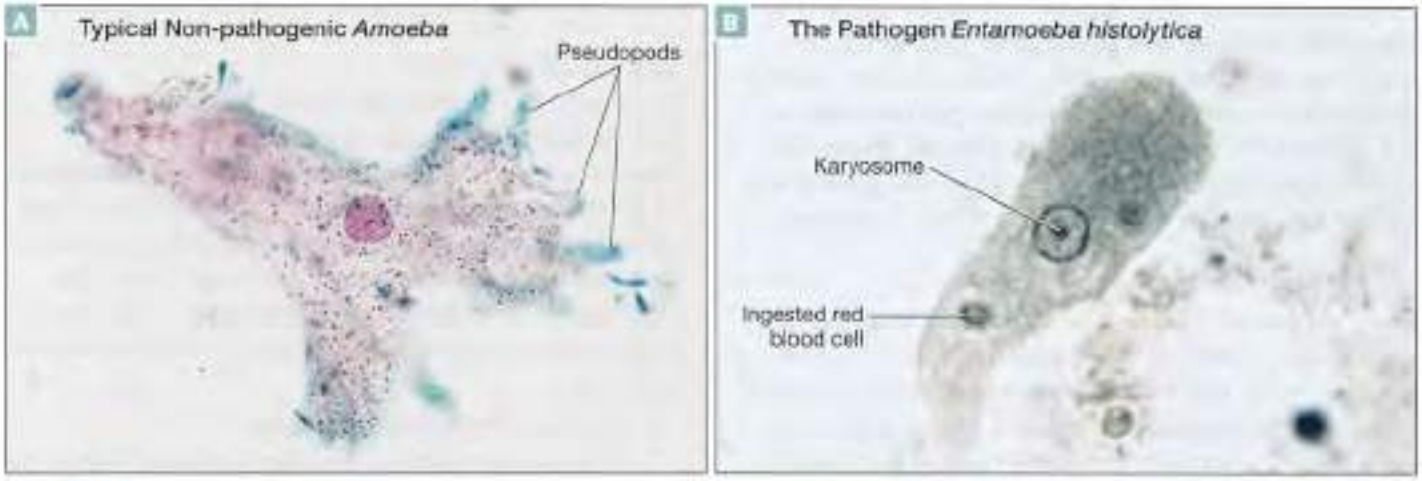


FIGURE 2.11 Animal-like Protists: Amoeboid Protozoans • (A) **Typical Non-pathogenic Amoeba:** This is a typical non-pathogenic *Amoeba*; here you can see numerous pseudopods, which are used for movement as well as engulfing prey. (Commercially prepared slide.) (B) ***Entamoeba histolytica:*** *Entamoeba histolytica* is the causative agent of amebic dysentery. Trophozoites range in size from 15 to 50 µm. Notice the small, central karyosome, the dark-staining, beaded chromatin at the periphery of the nucleus, and the ingested red blood cell, all of which are characteristic of *E. histolytica*. (Iron hematoxylin stain of fecal smear.)

developing countries. The largest risk factor seems to be contact with pigs, the suspected natural reservoir for this parasite.

B. coli exists in two forms: a vegetative trophozoite and a cyst. Infection develops upon ingesting the cyst stage, typically by consuming sewage

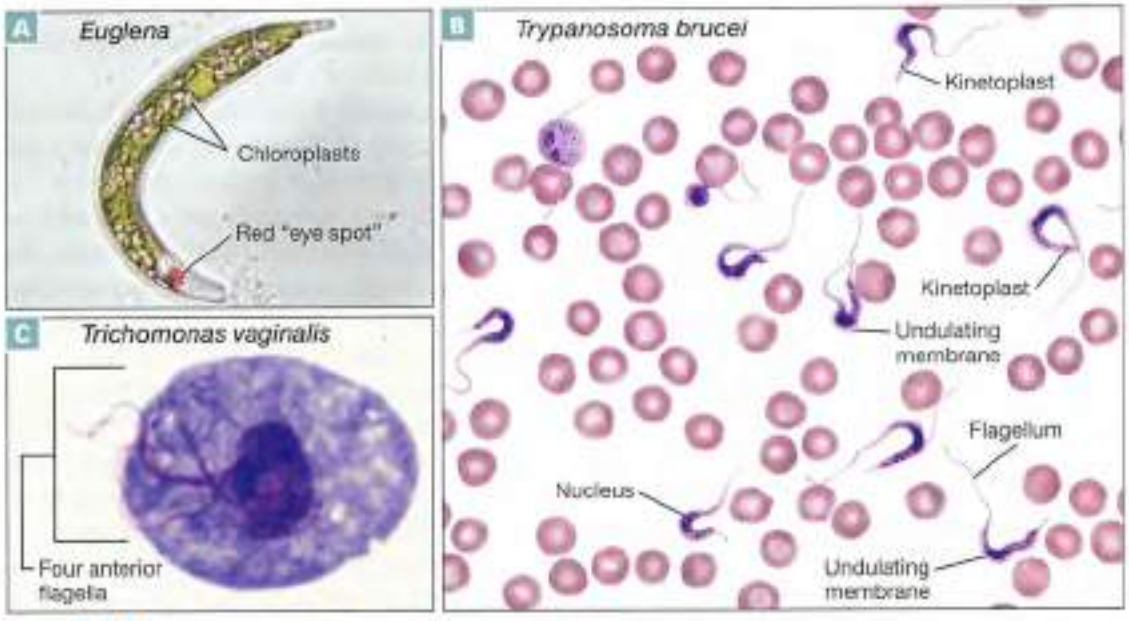


FIGURE **2.12** **Animal-like Protists: Flagellated Protozoans** • (A) ***Euglena:*** *Euglena* is a large genus of mixotrophic flagellates. Most species have chloroplasts and a red "eyespot" located in the colorless anterior of the cell. In this cell, a single flagellum also emerges from the anterior. (Wet mount from an environmental sample.) (B) ***Trypanosoma brucei:*** The central nucleus, posterior kinetoplast, and undulating membrane are visible in this commercially prepared blood smear of *Trypanosoma brucei*. (C) ***Trichomonas vaginalis:*** The *Trichomonas vaginalis* trophozoite is the only stage of the protist's life cycle and is 7–23 µm long by 5–15 µm wide. The four anterior flagella are visible here. (Commercially prepared slide.)

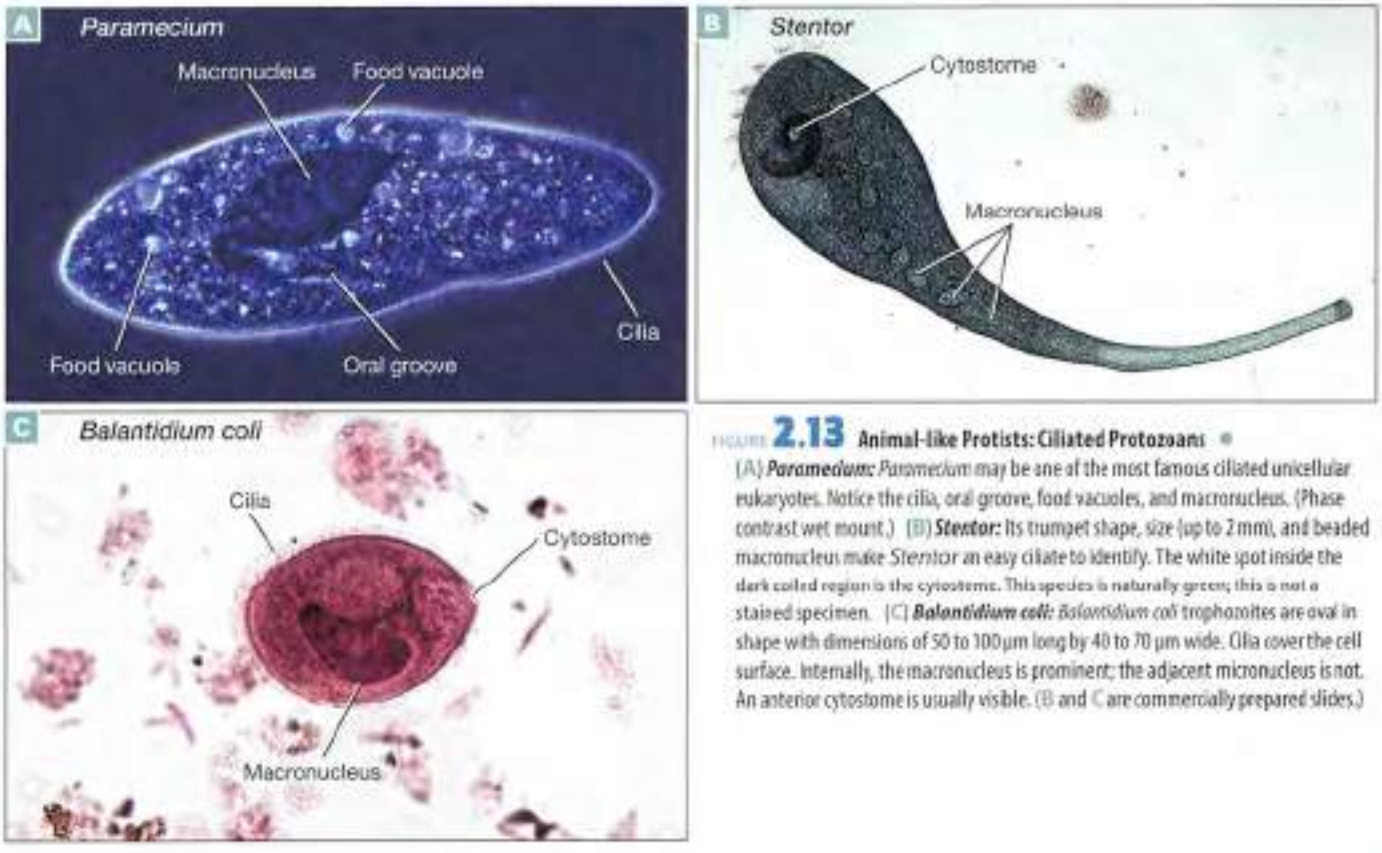


FIGURE **2.13** **Animal-like Protists: Ciliated Protozoans** • (A) ***Paramecium:*** *Paramecium* may be one of the most famous ciliated unicellular eukaryotes. Notice the cilia, oral groove, food vacuoles, and macronucleus. (Phase contrast wet mount.) (B) ***Stentor:*** Its trumpet shape, size (up to 2 mm), and beaded macronucleus make *Stentor* an easy ciliate to identify. The white spot inside the dark coiled region is the cytostome. This species is naturally green; this is not a stained specimen. (C) ***Balantidium coli:*** *Balantidium coli* trophozoites are oval in shape with dimensions of 50 to 100 µm long by 40 to 70 µm wide. Cilia cover the cell surface. Internally, the macronucleus is prominent; the adjacent micronucleus is not. An anterior cytostome is usually visible. (B and C are commercially prepared slides.)

contaminated food or water. The cysts make their way to the host's colon where they mature into trophozoites. Laboratory diagnosis involves identifying either cysts or the trophozoite in the patient's feces. Although most infections are asymptomatic, acute infection symptoms can include bloody and mucoid feces, nausea, vomiting, and abdominal tenderness. Diarrhea alternating with constipation may occur in chronic infections. The infection is treated with a combination drug therapy.

- **Spore-Forming Protozoans (Apicomplexa)**
  In their mature form, apicomplexans lack flagella, cilia, and pseudopodia; they instead move by gliding. Most apicomplexans are obligate intracellular parasites that have a complex life cycle involving more than one host and multiple tissues. For instance, *Plasmodium* species (Fig. 2.14) cause malaria and infect both liver and red blood cells of humans, and the gut and salivary glands of the insect vector, the female *Anopheles* mosquito. Another noteworthy apicomplexan is *Toxoplasma gondi*, which is often spread by cats and causes toxoplasmosis.

Plasmodium falciparum

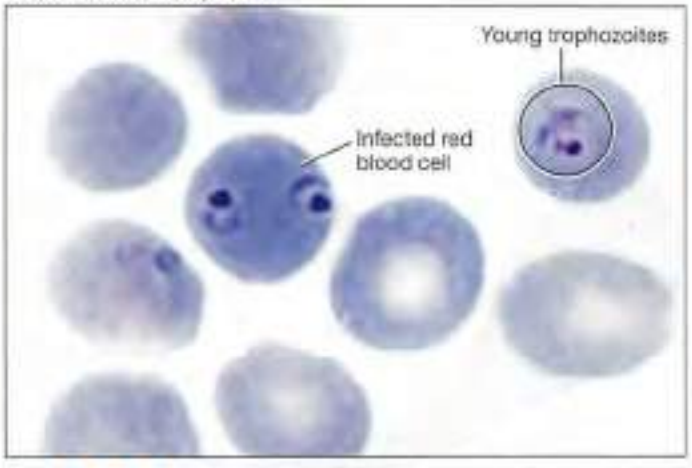


FIGURE **2.14** **Animal-like Protists: Apicomplexans** • *Plasmodium falciparum* is a causative agent of malaria. Shown here are young trophozoites, called "ring forms," within red blood cells (RBCs). One RBC has been infected by two trophozoites, a common occurrence with *P. falciparum*. (Commercially prepared slide.)

Clinical Applications

Medically speaking, aside from the kingdom Plantae, we find infectious agents among all the eukaryotic kingdoms. A basic understanding of eukaryotic cells, especially eukaryotic pathogens, is important for anyone entering a medically related field.

In the animal kingdom, we find **helminths** (parasitic roundworms and flatworms), which are typically transmitted to their host in a microscopic form (e.g., as larvae or eggs). In the kingdom Protista, we find a miscellaneous assortment of infectious agents that cause diseases ranging from the common sexually transmitted disease trichomoniasis to rare forms of **encephalitis** (inflammation of the brain) and malaria.

Among the fungi, we find select organisms that infect humans and other animals as either **true pathogens** (cause disease in normally healthy hosts) and/or as opportunistic pathogens. Fungal infections are called **mycoses**. These infections can range from a mere discomfort, such as athlete's foot (a type of "ringworm" infection), to potentially deadly forms of encephalitis caused by a variety of fungi such as *Cryptococcus*, *Coccidioides*, *Histoplasma*, and *Blastomyces*.

MATERIALS

- ❑ Compound light microscope
- ❑ Lens paper
- ❑ Lens-cleaning solution or 95% ethanol
- ❑ Immersion oil
- ❑ Lab coat and safety glasses if performing Part C
- ❑ Prepared slides (as available) of:
  - ☐ *Aspergillus* conidiophore (Part A)
  - ☐ *Candida albicans* (Part A)
  - ☐ *Penicillium* conidiophore (Part A)
  - ☐ *Rhizopus* sporangia and gametangia (Part A)
  - ☐ *Entamoeba histolytica* trophozoites (Part B)
  - ☐ *Trypanosoma* species trypomastigotes in blood (Part B)
  - ☐ *Trichomonas vaginalis* (Part B)
  - ☐ *Balantidium coli* trophozoites (Part B)
  - ☐ *Plasmodium* species (thick blood smear) (Part B)
  - ☐ Diatoms (centric and pennate) (Part B)

(continues)

MATERIALS (continued)

- ❑ Clean glass slides and cover glasses (Part C)
- ❑ Cytological stains (e.g., methylene blue, Lugol's iodine) (Part C)
- ❑ Cotton swabs (Part C)
- ❑ Methyl cellulose (Part C)
- ❑ Living cultures (as available) (Part C)

Protists:

- ☐ *Amoeba*
- ☐ *Chlamydomonas*
- ☐ *Euglena*
- ☐ *Paramecium*
- ☐ *Spirogyra*
- ☐ *Stentor*
- ☐ *Volvox*

Fungi:

- ☐ *Saccharomyces cerevisiae*

PROCEDURE

There are three parts in this lab. Parts A and B involve viewing commercially prepared slides of fungi and protists. Part C involves preparing and viewing wet mounts of living organisms. You may be asked to perform all or some of these parts. Your instructor will advise you as to which ones to do and which slides/specimens you should view.

PART A: Viewing Premade Slides of Fungi

For Steps 1–5 in this lab, use the premade slides in the following list or slides specified by your instructor.

- *Aspergillus* conidiophore: identify hyphae, conidiophores, and chains of conidia.
- *Candida albicans*: identify vegetative cells and budding cells.
- *Penicillium* conidiophore: identify hyphae, conidiophores, and chains of conidia.
- *Rhizopus* (sporangia and gametangia)
  - Sporangia: identify sproangiophores, sporangia, and spores.
  - Gametangia: identify progametangia, gametangia, young zygosporangia, and mature zygosporangia.

1. Obtain a microscope and place it on the table. Check that the stage is all the way down and that the scanning objective (4× objective) is in place.
2. Begin with a prepared slide. Clean it with a tissue if it is dirty, and then place it on the microscope stage. Center the specimen under the scanning objective.
3. Follow the instructions from the Lab Technique in Exercise 1, page 21, to bring the specimen into focus.
4. Scan the specimen until you're satisfied that you have seen everything in the list provided, or as specified by your instructor. Sketch what you see in the table provided in the data sheet at the end of this lab.
5. Repeat this process with as many slides as you have time for. Use the appropriate figures in this exercise to help you identify the organisms and their structures. *Note:* You may have to look at more than one slide to find the listed features for each organism. Your instructor will advise you of their expectations for this.

PART B: Viewing Premade Slides of Protists

Repeat the process described in Part A, but use the slides in the following list or slides your instructor specifies.

- *Balantidium coli* trophozoites: identify cilia, macronucleus, micronucleus (maybe), and cytostome.
- Diatoms: identify centric and pennate shapes, chloroplasts, oil droplets, and cell walls with sculpturing.
- *Entamoeba histolytica* trophozoites: identify pseudopods; nucleus, central karyosome, and ingested red blood cells.
- *Plasmodium falciparum* (or other species): identify red blood cells and ring stage of trophozoites.
- *Trichomonas vaginalis*: identify nucleus and flagella.
- *Trypanosoma* in blood: identify trypomastigotes with flagella, undulating membrane, kinetoplast, nucleus, and red blood cells.

PART C: Making Wet-Mount Slides of Select Eukaryotes

1. Prepare wet mounts of available specimens by following the procedural diagram in Figure 2.15. A drop of methyl cellulose may be added to the wet mount if you have fast swimmers.
2. Sketch what you see and record cellular dimensions in the table provided on the data sheet at the end of the exercise.
3. Repeat with as many specimens as time permits.

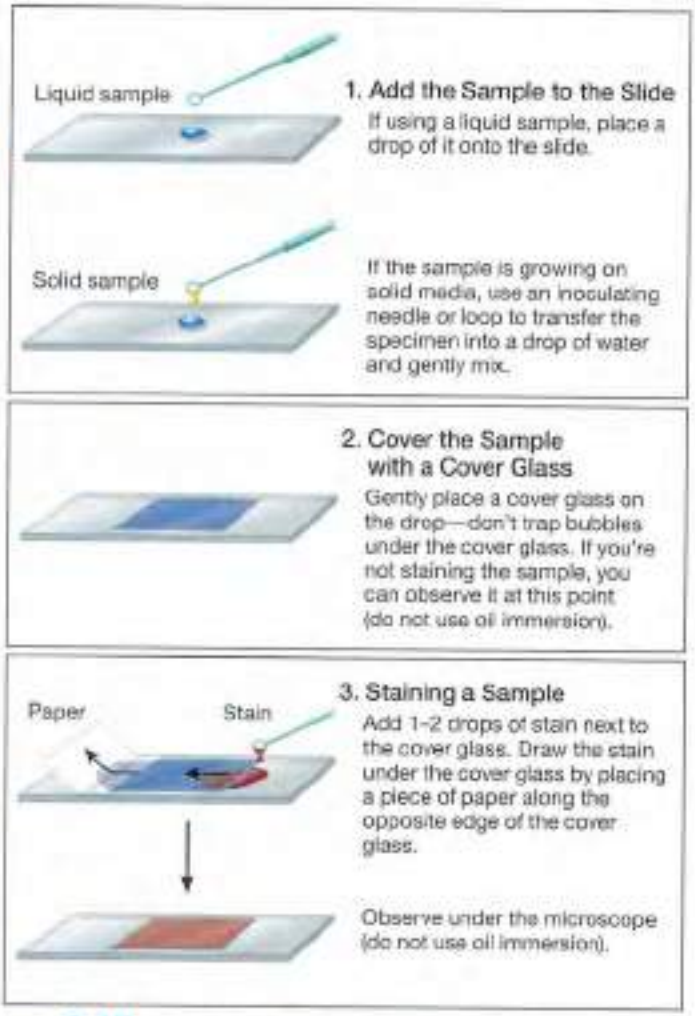

Liquid sample
1. Add the Sample to the Slide
If using a liquid sample, place a drop of it onto the slide.
Solid sample
If the sample is growing on solid media, use an inoculating needle or loop to transfer the specimen into a drop of water and gently mix.
2. Cover the Sample with a Cover Glass
Gently place a cover glass on the drop—don't trap bubbles under the cover glass. If you're not staining the sample, you can observe it at this point (do not use oil immersion).
Paper
Stain
3. Staining a Sample
Add 1–2 drops of stain next to the cover glass. Draw the stain under the cover glass by placing a piece of paper along the opposite edge of the cover glass.
Observe under the microscope (do not use oil immersion).

FIGURE **2.15** **Preparing a Wet-Mount Slide** • The preparation may be stained or not, but staining will eventually kill the organisms. Of course, so will drying. You can expect a wet mount to last only 15–20 minutes.

Specimens that will not be stained:

- *Amoeba*: identify nucleus, pseudopods, and ingested material.
- *Chlamydomonas*: identify chloroplast, flagella, and stigma.
- *Euglena*: identify chloroplast, flagellum, eyespot.
- *Paramecium*: identify macronucleus, cilia, oral groove, and contractile vacuole.
- *Spirogyra*: identify nuclei, spiral chloroplasts, and cell wall.
- *Stentor*: identify cilia, beaded macronucleus, and cytostome.
- *Volvox*: identify colony and daughter colonies.

The following sample requires staining with methylene blue or Lugol's iodine:

- *Saccharomyces cerevisiae*: identify vegetative cells, budding cells, and nuclei.

PART D: Clean Up

1. When you are finished observing the specimens, use lens paper to remove oil from the oil-immersion lens (if used). Also, check the 40× lens for oil and clean it if necessary.
2. Return all lenses and adjustments to their storage positions before putting the microscope away.
3. Return prepared slides to their proper storage box. Dispose of wet-mount slides as directed by your instructor.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 2

DATA SHEET

Microscopic Examination of Eukaryotic Microbes

During the Lab

1 Fill in the table for each eukaryotic microbe you observe.

Organism (indicate if wet-mount or prepared slide)	Sketch (include magnification and stain, if any)	Identifying Characteristics (list only those you observed)

(continues)

Organism (Indicate if wet-mount or prepared slide)	Sketch (include magnification and stain, if any)	Identifying Characteristics (list only those you observed)

2 What features did the cells you observed have in common? How were they different?

3 If you observed the same organism on a prepared slide and a wet mount, how did the images compare?

Lab Related Questions

1 Which of the following could **not** apply to a eukaryotic organism that is unicellular, non-photosynthetic, and ciliated?

a. Protists
b. Protozoan
c. *Balantidium coli*
d. Parasite
e. Mastigophora

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 2
DATA SHEET
(continued)

2 Which features apply to fungi? (Select all that apply.)

a. Saprobes
b. Include multicellular organisms
c. Include unicellular organisms
d. Photosynthetic
e. Includes pathogens
f. Includes prokaryotes
g. Can reproduce sexually and/or asexually
h. Includes protists

3 The most common sexually transmitted eukaryotic infection is _____.

a. Toxoplasmosis
b. Trichomoniasis
c. Giardiasis
d. Gonorrhea
e. Trypanosomiasis

4 Organize these numerically from most specific (1) to least specific (8).

_____ Domain _____ Phylum
_____ Class _____ Genus
_____ Family _____ Species
_____ Kingdom _____ Order

5 Fungal infections are called _____.

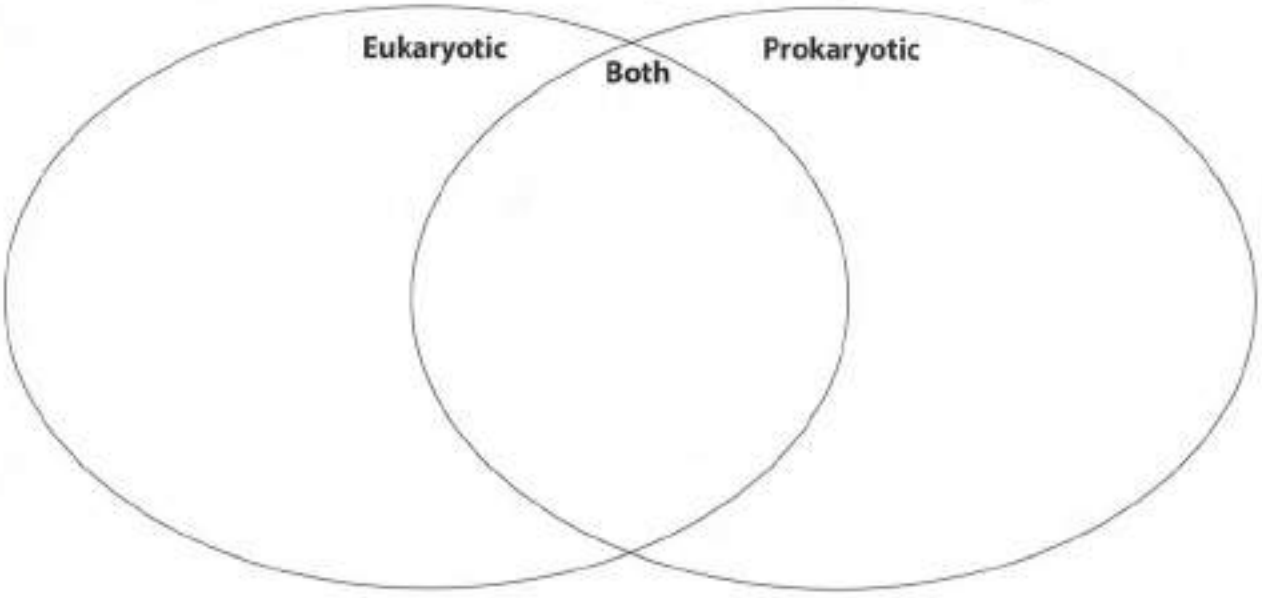
6 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ In their mature form, apicomplexans move by pseudopods.
_____ Protists are eukaryotic cells and include animal-like, plant-like, and fungi-like organisms.
_____ *Rhizopus* is an example of a unicellular fungus.
_____ Protists are grouped based on their form of sexual reproduction.
_____ Diatoms are an example of a plant-like protist.
_____ Yeast are the only unicellular fungus.
_____ Prokaryotic cells are typically smaller than eukaryotic cells.

7 Select all that apply to an opportunistic pathogen:

a. Include fungi.
b. Can be unicellular.
c. Are often photosynthetic.
d. An example is *Candida albicans*.
e. Tend to affect a healthy host.

8 Fill in the Venn diagram with the nine listed statements as they relate to prokaryotic and/or eukaryotic cells.



Can be pathogenic	Nucleus absent	Domain Archaea
Domain Bacteria	Fungi	Can be unicellular
Can be multicellular	Nucleated	Ribosomes present

Critical Thinking

1 As compared to the availability of drugs to treat prokaryotic infections, the pharmacopeia of drugs that can safely and effectively treat eukaryotic infections is fairly limited. Why is it especially challenging to develop safe anti-protozoan drugs for human and/or animal use?

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 2
DATA SHEET
(continued)

2 The endosymbiotic theory describes how eukaryotic cells evolved when an ancient ancestor engulfed (endocytosed) prokaryotic cells. The engulfed cells and the host cell had a symbiotic relationship—a mutually beneficial relationship. Over time, the cells in this symbiotic relationship increasingly relied on one another to the point that they became inseparable. Eventually those engulfed cells that were photosynthetic became chloroplasts and those that were non-photosynthetic became mitochondria. Based on the figures and descriptions in this laboratory, which cells do you think were engulfed first, photosynthetic cells or non-photosynthetic cells? **Provide support for your answer.**

— Lab Notes —

SECTION 2

Aseptic Technique and Bacterial Growth and Enumeration

In This Section

EXERCISE 3 Common Aseptic Transfer and Inoculation Methods 49

EXERCISE 4 Streak Plate Isolation Methods 69

EXERCISE 5 Spread Plate Isolation Method 81

EXERCISE 6 Colony Morphology 87

EXERCISE 7 Growth Patterns on Slants 101

EXERCISE 8 Growth Patterns in Broth 107

EXERCISE 9 Ubiquity of Microorganisms 111

EXERCISE 10 Standard Plate Count 119

— Lab Notes —

EXERCISE 3

Common Aseptic Transfer and Inoculation Methods

After doing this exercise, you should be able to:

1. Identify the various formats of growth media and properly inoculate them.
2. State the goals of aseptic methods.
3. Correctly apply aseptic methods while handling bacterial cultures and media.
4. Describe the necessary aspects of aseptic culture transfer.
5. Compare complex media to defined media and provide examples of each.
6. Define the terms *mixed culture* and *pure culture*.
7. Describe ways to minimize risks when working with BSL-2 agents.
8. Properly label microbial samples.

Background

Growing bacteria is a basic procedure in any microbiology lab. We start by **inoculating** (transferring) some bacteria into a **growth media**, a liquid, solid, or semisolid substance that provides necessary nutrients for specimen growth. Growth media (or simply, media) come in hundreds of formulations. The recipe used depends on the goal, from generalized growth to bacterial species differentiation and/or isolation. Here we'll review some basics about growth media and the aseptic methods for inoculating such media. In later laboratory exercises, we'll discuss more sophisticated inoculation methods used for species isolation (e.g., the streak plate isolation) and the collection of media used for differentiating bacterial species from one another.

In general, growth media have either a defined or complex formulation. **Defined media** (sometimes called synthetic media) get their name from the fact that each organic and inorganic ingredient is precisely known and quantified, which means these media are accurately reproducible. Glucose minimal salts media is an example. In contrast, **complex media** (also called enriched media) contain ingredients such as milk proteins, blood, or yeast extracts, less precisely defined organic nutrients. Most pathogens are fastidious (require complex nutrient sources) and will only grow on enriched media. In this lab exercise, we'll use a complex media called **nutrient broth**, which contains peptone (a solution of partially broken-down proteins) and beef extract.

Two Important Media Formats: Liquid and Solid

As their name suggests, **broth media** are liquids. Liquid media are great for quickly growing large quantities of bacteria. This is because high-volume batches can be readily prepared and easily inoculated. However, sometimes a solid media format is required.

To make solid media, we simply add **agar** to liquid media. Agar is purified from seaweed. It has the same solidifying effect as gelatin, but it's better for most bacterial growth applications because it remains solid at standard incubation temperatures and most pathogens can't digest it. The thickened media can be poured into sterile petri plates to make **agar plates**, upright test tubes to make **agar deeps**, or tilted test tubes to make **agar slants** (Fig. 3.1).

Plated media are used for isolating species, differential testing, and quantifying bacterial levels. We won't use plated media in this lab exercise, but we'll use them later. Agar slants are used for a variety of biochemical tests and, because they take up less space than plated media, they're useful for maintaining stock cultures. Most deeps are used to determine specific bacterial metabolic properties. No matter what media we use, each requires aseptic inoculation.

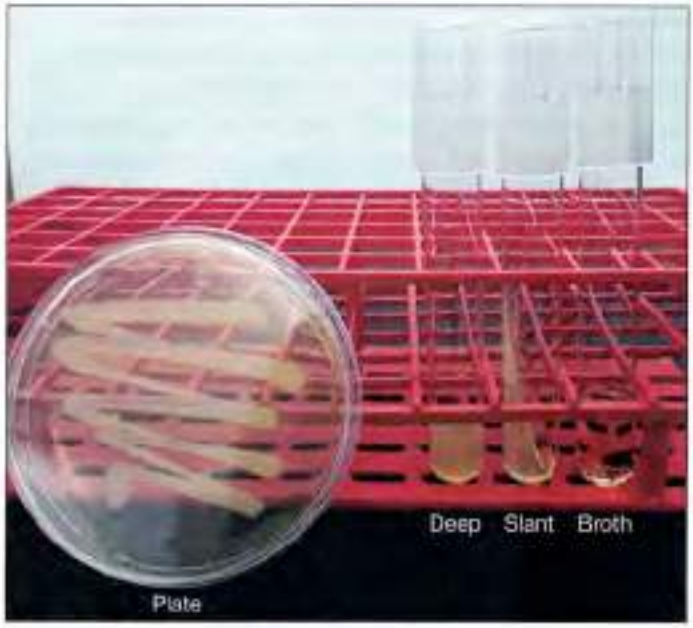


FIGURE **3.1** Media Formats •

Aseptic Inoculation Methods

When growing bacteria in the lab, we start with a **culture**, a sample containing bacteria. If the sample contains only one species, we call it a **pure culture**. In contrast, a **mixed culture** contains more than one species. No matter what type of culture we start with, when transferring it to new media we must avoid contaminating it with additional microbes from the environment and we must avoid exposing ourselves and our surroundings to the microbes in our sample. To achieve this, we use **aseptic methods** (*a* = without; *septic* = contamination), which involve precise sample handling methods combined with using sterile media and sterile inoculating tools.

Sterile Media

One goal of aseptic methods is to avoid contaminating our culture with unwanted microbes. Thus, the growth media must be sterile before it's inoculated. There are a few ways to sterilize media, but the most common approach is to use an **autoclave**, a chamber that steam heats a sample under pressure. In addition to sterilizing the media, anything that will directly contact the sample must also be sterile. This includes the interior of the plastic or glass containers that hold the media. There are a couple of ways to do this: the media can be mixed and poured into the tubes and then immediately autoclaved so the media and glassware are simultaneously sterilized, or the sterilized media can be poured into presterilized petri plates and other plastic or glassware.

Sterile Inoculating Tools

All inoculating tools used to transfer the sample to the sterile media must also be sterile. There are many inoculating tools available, but in this lab exercise, we'll use a **wire inoculating needle** and a **wire inoculating loop** to pick up small amounts of a culture and transfer it to our media (Fig. 3.2). An inoculating loop has a handle that holds a thin tungsten, platinum, or nichrome wire twisted into a small loop at the end. An inoculating needle is basically the same as a loop, except the wire is straight. To sterilize the loop or needle, the tool's wire filament is inserted into an incinerator or Bunsen burner flame until the filament glows red-hot (Fig. 3.3). If presterilized disposable inoculating loops or needles are used, then the incineration/flame sterilization process is skipped. Sometimes other inoculating tools like pipettes are used; these are covered in Appendices C and D.

Protecting Yourself and Others

When properly performed, aseptic methods help you avoid sample contamination, but more importantly these techniques protect you and others from exposure to potential pathogens. You must do two things to accomplish this. First, limit how much you expose your sample to the environment. Second, limit sample agitation to avoid the formation of airborne particles (e.g., aerosols and droplets) when handling the culture. We'll delve into each process as we walk through the inoculation procedures. For now, here is a list of things to keep in mind:

- **Pay attention to your environment.** Your workspace should be clutter free. Never perform transfers over your books and papers because you may unknowingly contaminate them.
- **Thoroughly label all media.** Arrange all media in advance and clearly label them with your name, the date, the type of medium (e.g., nutrient broth, or NB for short), and the bacterium's name (or the sample name, such as Unknown #3) (Fig. 3.4). Never label lids. Instead, label the body of your tubes with tape, paper held on with a rubber band, or by writing directly on the glass. For plastic plates, write directly on the base (not the lid). Your instructor will tell you their labeling preferences. Whatever labeling method used, be sure you position labels so they don't obscure your view of the media.
- **Keep test tubes in a test-tube rack.** Never lay tubes on the table; they may leak or roll off (Fig. 3.5).
- **Take your time.** You are handling potentially dangerous microbes. Working at a frenzied pace leads to accidents.

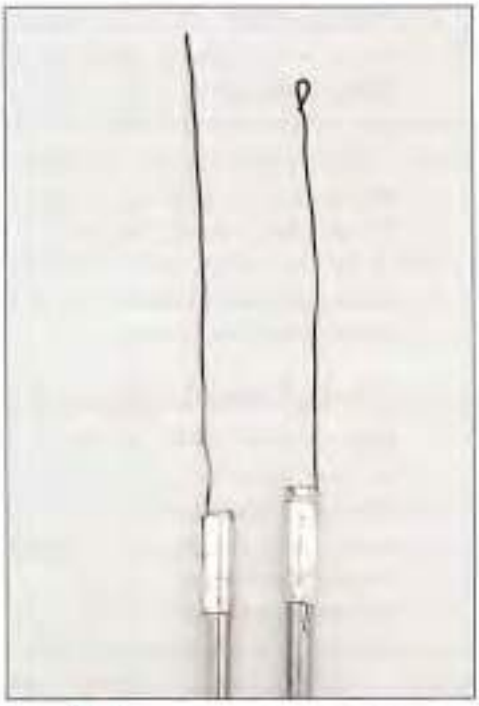


FIGURE **3.2** Wire Inoculating Needle and Loop

- **Never hold a tube by its cap.** Caps are designed to come off and are therefore a bad way to hold a sample. Plus, during incubation, caps are generally kept loose to allow for sample aeration. This is true for push caps and screw caps.
- **Use your dominant hand to hold inoculating tools.** With a relaxed grasp, hold your inoculating tool like a pencil (Fig. 3.5).

A Special Note on BSL-2 Agents

The primary concern with BSL-2 organisms is the formation of aerosols, which can lead to infection if inhaled and/or environmental contamination. Aerosols are problematic because we are generally unaware of their production, and they remain suspended in the air long after the procedure is complete. Because of the risks associated with BSL-2 pathogens, most college teaching laboratories have reduced, or even eliminated, the use of BSL-2 organisms. We've followed suit in this lab manual while trying to honor the fact that many people taking an introductory microbiology course want to work in health-care settings, places where BSL-2 organisms are unavoidable. As such, we often include "optional" BSL-2 bacteria in the exercises and simply leave the decision up to your instructor as to when (or if) you will use these agents.

Even if you do not use BSL-2 agents in the lab, most microbiology instructors will train you to protect yourself and others against these microbes. Unless your instructor

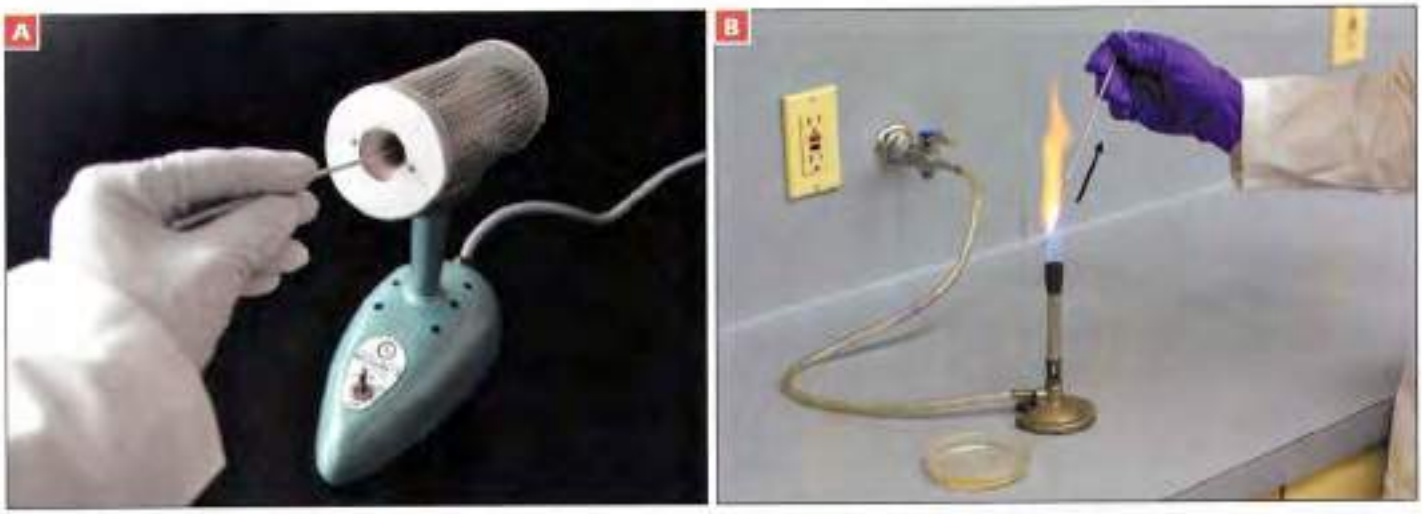


FIGURE **3.3** **Using an Incinerator or Bunsen Burner to Sterilize Inoculating Tools** • (A) An incinerator or (B) a Bunsen burner can be used to sterilize inoculating loops and needles. In either case, the full length of the inoculating tool's wire filament must be heated until red-hot, which takes about 3–7 seconds.

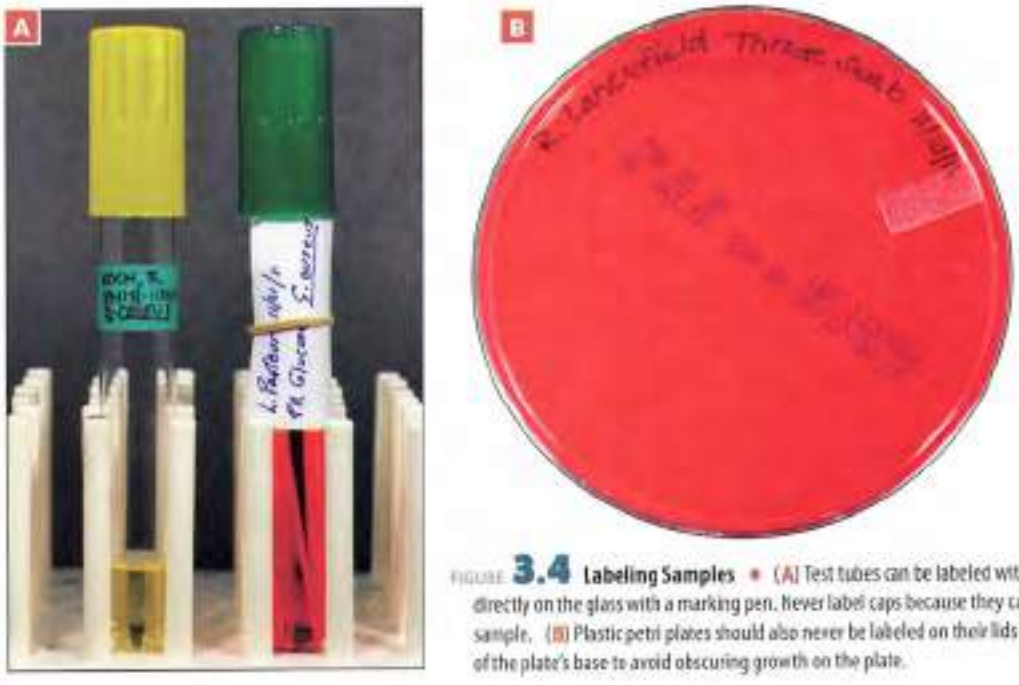


FIGURE **3.4** **Labeling Samples** • (A) Test tubes can be labeled with tape, paper labels, or directly on the glass with a marking pen. Never label caps because they can be separated from the sample. (B) Plastic petri plates should also never be labeled on their lids. Instead, label at the edge of the plate's base to avoid obscuring growth on the plate.

says otherwise, you should assume you are to use BSL-2 precautions for lab exercises that involve live bacterial specimens.

Throughout this manual, we emphasize techniques that minimize aerosol production, but they are even more essential when handling BSL-2 organisms. Your instructor will have specific guidelines on how to handle BSL-2 organisms. Precautions may include any (or all) of the following depending on the exercise and the equipment available. Whatever the dictated guidelines are, for your own health (and the health of others), take every precaution seriously.

- Performing tests in a biosafety cabinet is often recommended, but not all teaching laboratories have these installed.

FIGURE **3.5** **A Microbiologist at Work** • Materials are neatly positioned and the work space is not cluttered. The Bunsen burner is accessible, but not so close as to be a major fire hazard. Test tubes are never laid on a table, instead they should be stored upright in a test-tube rack. Notice how the microbiologist holds the loop like a pencil and their little finger is holding the tube's cap—the cap is not on the table.

- An electric incinerator can reduce aerosol exposure because any aerosols that may develop while flaming inoculating tools do not escape the incinerator's chamber.
- Using sterile disposable loops/needles or sterile wooden sticks that don't require flaming after use can also help reduce aerosol formation. However, this approach increases the burden of biological waste management.
- Always wear lab safety gear (lab coat, gloves, and eye protection) and lab appropriate attire as dictated by your instructor (e.g., closed-toe shoes, long pants/skirt, shirt that covers your shoulders and the entirety of your torso).
- Clearly identify BSL-2 organisms. Red caps on tube cultures or BSL-2 labels on plate cultures can be helpful.

Inoculating Various Non-plated Media

In this lab, you'll use broth, slant, and plated stock cultures to inoculate various forms of non-plated media. Refer to the steps and procedural figures in the following Lab Techniques to help you understand each method. Always put on your safety gear before performing any procedure involving live microbes.

Transferring Culture from a Broth to Another Broth *(continues)*

Figure 3.6 on pages 54–55 provides a photographic review of each step in the following process.

1. **Check the Loop:** Ensure your inoculating loop has a fully closed circle at the end, if not, pinch it closed.
2. **Mix the Sample:** Mix the broth culture with a vortex mixer or by drumming your fingers along the tube. Do not mix so vigorously that broth enters the cap. Once it's mixed, loosen the cap and return the tube to the rack.
3. **Sterilize the Loop:** Hold the inoculating loop in your dominant hand like a pencil and heat the length of the wire filament until each section glows orange. Once the filament is sterilized, do not put it down and don't touch it to any surface.
4. **Open the Stock Sample:** Pick up the culture tube with your free hand. Then, using the little finger of the hand holding your loop, remove the tube's cap. Hold the cap with your pinky; do not put the cap down.
5. **Flame the Stock Tube's Opening:** Holding the open tube at a slight angle, quickly pass the mouth of the tube through the flame a few times (don't let broth slosh out of the tube). If you're using an electric incinerator, hold the mouth of the tube near the entrance of the incinerator.
6. **Collect the Sample with a Loop:** Holding the tube at an angle, dip the inoculating loop into the broth and then carefully withdraw the loop. Avoid touching any part of the tube with your loop. At this point, there should be a visible film of broth in your loop.
7. **Re-flame the Stock Tube's Opening:** While holding your loop hand still, flame the mouth of the open tube, replace the cap, and return the tube to the rack.
8. **Open the Sterile Broth:** With your free hand, pick up the sterile broth tube and repeat the process of removing the cap with your loop hand's little finger and flaming the lip of the open tube.
9. **Inoculate the Sterile Broth:** Dip the loop into the sterile broth and gently swirl the loop.
10. **Remove the Loop:** Before removing the loop, tap the loop's face on the inside of the tube to remove the film within the loop. Be persistent, if you flame the loop with the film present you'll produce aerosols.
11. **Flame the Newly Inoculated Tube's Opening:** Keeping your loop hand still, flame the tube's opening (don't let broth slosh out of the tube), replace the cap, and return the tube to the rack. Sample caps should be kept loose for incubation, but not so loose that they will easily fall off.
12. **Re-sterilize the Loop:** Before putting the loop down, re-flame the filament to sterilize it.

Transferring Culture from a Broth to Another Broth *(continued)*

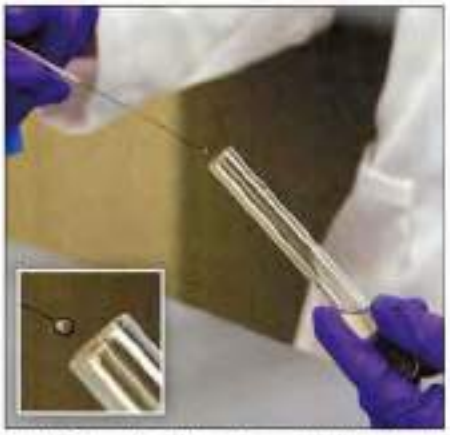
1. **Check the Loop:** Ensure your inoculating loop has a fully closed circle at the end, if not, pinch it closed.

2. **Mix the Sample:** Mix the broth culture with a vortex mixer or by drumming your fingers along the tube. Do not mix so vigorously that broth enters the cap. Once it's mixed, loosen the cap and return the tube to the rack.

3. **Sterilize the Loop:** Hold the inoculating loop in your dominant hand like a pencil and heat the length of the wire filament until each section glows orange. Once the filament is sterilized, do not put it down and don't touch it to any surface.

4. **Open the Stock Sample:** Pick up the culture tube with your free hand. Then, using the little finger of the hand holding your loop, remove the tube's cap. Hold the cap with your pinky; do not put the cap down.

5. **Flame the Stock Tube's Opening:** Holding the open tube at a slight angle, quickly pass the mouth of the tube through the flame a few times (don't let broth slosh out of the tube). If you're using an electric incinerator, hold the mouth of the tube near the entrance of the incinerator.

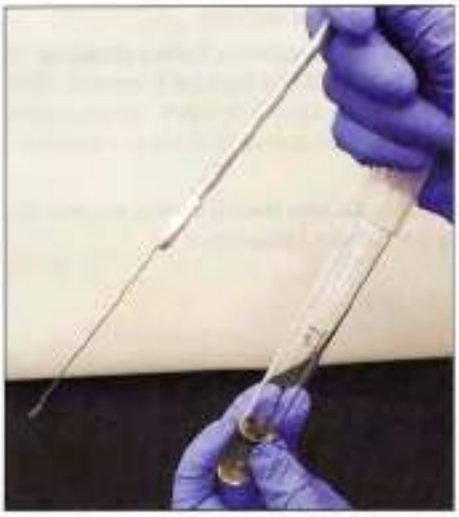


6. **Collect the Sample with a Loop:** Holding the tube at an angle, dip the inoculating loop into the broth and then carefully withdraw the loop. Avoid touching any part of the tube with your loop. At this point, there should be a visible film of broth in your loop.

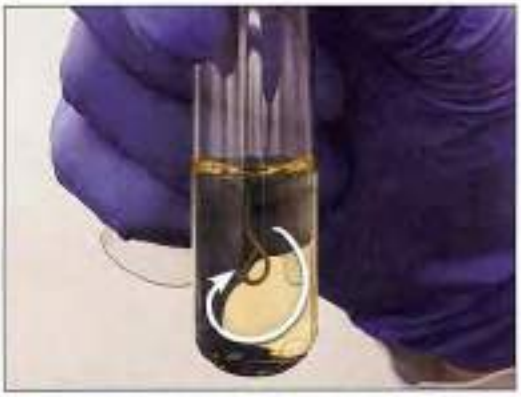
FIGURE **3.6** **Transferring Culture from a Broth to Another Broth** • *(continued)*

Transferring Culture from a Broth to Another Broth *(continued)*

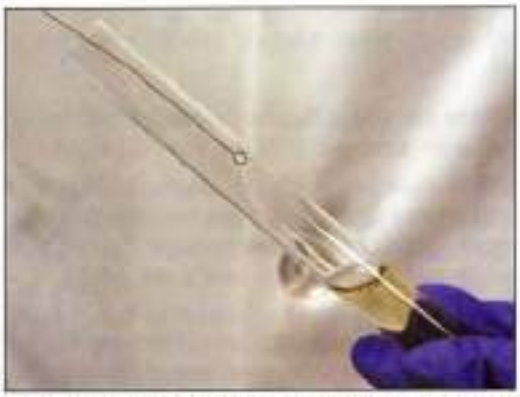
7 Re-flame the Stock Tube's Opening: While holding your loop hand still, flame the mouth of the open tube, replace the cap, and return the tube to the rack.



8 Open the Sterile Broth: With your free hand, pick up the sterile broth tube and repeat the process of removing the cap with your loop hand's little finger and flaming the lip of the open tube.



9 Inoculate the Sterile Broth: Dip the loop into the sterile broth and gently swirl the loop.



10 Remove the Loop: Before removing the loop, tap the loop's face on the inside of the tube to remove the film within the loop. Be persistent, if you flame the loop with the film present you'll produce aerosols.

11 Flame the Newly Inoculated Tube's Opening: Keeping your loop hand still, flame the tube's opening (don't let broth slosh out of the tube), replace the cap, and return the tube to the rack. Sample caps should be kept loose for incubation, but not so loose that they will easily fall off.

12 Re-sterilize the Loop: Before putting the loop down, re-flame the filament to sterilize it.

FIGURE **3.6** *(continued)* **Transferring Culture from a Broth to Another Broth** •

Transferring Culture from a Broth to an Agar Slant

The steps in this process are very similar to what you use to inoculate from a broth to another broth (Fig. 3.6). The main difference is Step 9, where instead of dipping the **inoculum** (collected sample) into a broth, you'll perform a fishtail inoculation (Fig. 3.7).

1. **Check the Loop:** Ensure your inoculating loop has a fully closed circle at the end. If it's not, pinch it closed.
2. **Mix the Sample:** Mix the broth culture with a vortex mixer or by drumming your fingers along the tube. Do not mix so vigorously that broth enters the cap. Once it's mixed, loosen the cap and return the tube to the rack.
3. **Sterilize the Loop:** Hold the inoculating loop in your dominant hand like a pencil and heat the length of the wire filament until each section glows orange. Once the filament is sterilized, do not put it down and don't touch it to any surface.
4. **Open the Stock Sample:** Pick up the culture tube with your free hand. Then, using the little finger of the hand holding your loop, remove the tube's cap. Hold the cap with your pinky; do not put the cap down.
5. **Flame the Stock Tube's Opening:** Holding the open tube at a slight angle, quickly pass the mouth of the tube through the flame a few times (don't let broth slosh out of the tube). If you're using an electric incinerator, hold the mouth of the tube near the entrance of the incinerator.
6. **Collect the Sample with a Loop:** Holding the tube at an angle, dip the inoculating loop into the broth and then carefully withdraw the loop. Avoid touching any part of the tube with your loop. At this point, there should be a visible film of broth in your loop.
7. **Re-flame the Stock Tube's Opening:** While holding your loop hand still, flame the mouth of the open tube, replace the cap, and return the tube to the rack.
8. **Open the Sterile Slant:** With your free hand, pick up the sterile slant tube and repeat the process of removing the cap with your loop hand's little finger and flaming the lip of the open tube.
9. **Fishtail Inoculation of the Sterile Slant:** Starting at the bottom of the slant, gently drag the inoculating loop across the slant surface in a zigzag pattern until you reach the top of the slant (Fig 3.7). ***Note:*** If you use too much pressure, you'll gouge the agar.
10. **Remove the Loop:** Carefully remove the loop. Avoid catching it on the lip of the tube.
11. **Flame the Newly Inoculated Tube's Opening:** Keeping your loop hand still, flame the tube's opening, replace the cap, and return the tube to the rack. Sample caps should be kept loose for incubation, but not so loose that they will easily fall off.
12. **Re-sterilize the Loop:** Before putting the loop down, re-flame the filament to sterilize it.

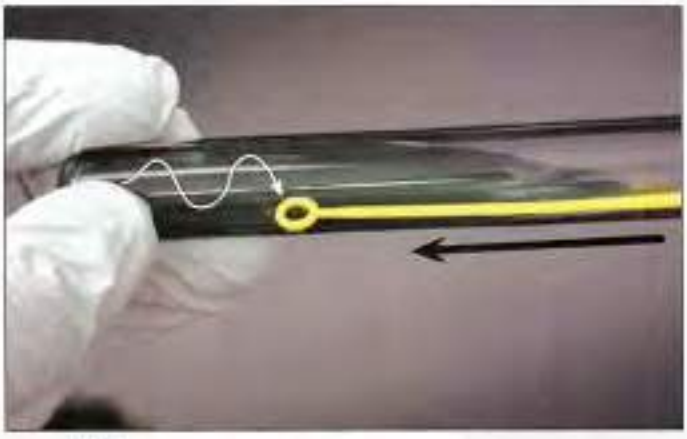
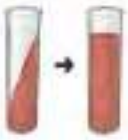


FIGURE **3.7** **A Fishtail Inoculation** • A zigzag (or fishtail) inoculation is used to inoculate an agar slant. (A disposable loop was used in this photo.)

Transferring Culture from a Slant to a Broth

The primary difference for this procedure versus the others is you'll collect some sample off a slant culture and introduce it to a broth.

1. **Check the Loop:** Ensure your inoculating loop has a fully closed circle at the end, if not, pinch it closed.
2. **Loosen the Cap on the Slant Sample:** Loosen, but don't remove, the cap on the slant culture and return the tube to the rack.
3. **Sterilize the Loop:** Hold the inoculating loop in your dominant hand like a pencil and heat the length of the wire filament until each section glows orange. Once the filament is sterilized, do not put it down and don't touch it to any surface.
4. **Open the Stock Sample:** Pick up the culture tube with your free hand. Then, using the little finger of the hand holding your loop, remove the tube's cap. Hold the cap with your pinky; do not put the cap down.
5. **Flame the Stock Tube's Opening:** Holding the open tube at a slight angle, quickly pass the mouth of the tube through the flame a few times. If you're using an electric incinerator, hold the mouth of the tube near the entrance of the incinerator.
6. **Collect the Sample with a Loop:** Holding the tube at an angle, use the inoculating loop to collect a pin-head amount of the culture growing on the slant's surface. Be very gentle so that you do not gouge the agar. Then, withdraw the loop. Avoid touching any part of the tube with it.
7. **Re-flame the Stock Tube's Opening:** While holding your loop hand still, flame the mouth of the open tube, replace the cap, and return the tube to the rack.
8. **Open the Sterile Broth:** With your free hand, pick up a tube of sterile broth and repeat the process of removing the cap with your loop hand's little finger and flaming the lip of the open tube.
9. **Inoculate the Sterile Broth:** Dip the loop into the sterile broth and gently swirl the loop.
10. **Remove the Loop:** Before removing the loop, tap the loop's face on the inside of the tube to remove the film within the loop. Be persistent, if you flame the loop with the film present you'll produce aerosols.
11. **Flame the Newly Inoculated Tube's Opening:** Keeping your loop hand still, flame the tube's opening (don't let the broth slosh out of the tube), replace the cap, and return the tube to the rack. Sample caps should be kept loose for incubation, but not so loose that they will easily fall off.
12. **Re-sterilize the Loop:** Before putting the loop down, re-flame the filament to sterilize it.

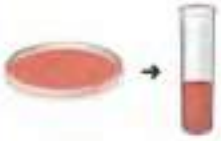


Transferring Culture from a Slant to a Deep

The steps in this process are very similar to the prior inoculations; the main difference is you will use an inoculating needle to introduce the sample into an agar deep (Fig. 3.8).

1. **Check the Needle:** Ensure your inoculating needle is reasonably straight, if not, straighten the wire. ***Caution!*** Be gentle! The wire easily pulls out of the handle.
2. **Loosen the Cap on the Slant Sample:** Loosen, but don't remove, the cap on the slant culture and return the tube to the rack.
3. **Sterilize the Inoculating Needle:** Hold the inoculating needle in your dominant hand like a pencil and heat the length of the wire filament until each section glows orange. Once the filament is sterilized, do not put it down and don't touch it to any surface.
4. **Open the Stock Sample:** Pick up the culture tube with your free hand. Then, using the little finger of the hand holding your needle, remove the tube's cap. Hold the cap with your pinky; do not put it down.
5. **Flame the Stock Tube's Opening:** Holding the open tube at a slight angle, quickly pass the mouth of the tube through the flame a few times. If you're using an electric incinerator, hold the mouth of the tube near the entrance of the incinerator.
6. **Collect the Sample with an Inoculating Needle:** Holding the tube at an angle, gently collect a small amount of culture onto the needle and carefully withdraw it. Avoid touching any part of the tube with your needle.
7. **Re-flame the Stock Tube's Opening:** While holding your needle hand still, flame the mouth of the open tube, replace the cap, and return the tube to the rack.
8. **Open the Sterile Agar Deep:** With your free hand, pick up the sterile agar deep tube and repeat the process of removing the cap with your needle hand's little finger and flaming the lip of the open tube.
9. **Stab Inoculate the Sterile Agar Deep:** In as straight a line as possible, slowly stab the middle of the agar deep until the needle is as close to the bottom of the agar as possible without having the handle touch the agar (Fig. 3.8).
10. **Remove the Needle:** Carefully remove the needle. Avoid catching it on the lip of the tube.
11. **Flame the Newly Inoculated Tube's Opening:** Keeping your needle hand still, flame the tube's opening, replace the cap, and return the tube to the rack. Sample caps should be kept loose for incubation, but not so loose that they will easily fall off.
12. **Re-sterilize the Needle:** Before putting the needle down, re-flame the filament to sterilize it.

FIGURE **3.8** **Stab Inoculation of an Agar Deep** • Agar deeps are stab inoculated with a needle. Use as straight a motion as possible to inoculate the middle of the agar.



Transferring Culture from a Plate to a Broth

In this process, you will use an inoculating loop to pick a colony from an agar plate culture and introduce it into agar broth. A **colony** is a visible collection of cells that are genetically alike. Therefore, if you inoculate a single colony into your media and do not introduce any contaminants into the sample, your resulting cultures should be pure. Whenever you open the plate, be sure you hold the lid like a clam shell. You can either set the plate on the table or hold the plate in your hand to do this (Fig. 3.9).

1. **Check the Loop:** Ensure your inoculating loop has a fully closed circle at the end, if not, pinch it closed.
2. **Loosen the Cap on the Sterile Broth Tube You'll Inoculate:** Loosen, but don't remove, the cap on the tube of broth you'll inoculate and return the tube to the rack.
3. **Sterilize the Loop:** Hold the inoculating loop in your dominant hand like a pencil and heat the length of the wire filament until each section glows orange. Once the filament is sterilized, do not put it down and don't touch it to any surface. Allow the loop to cool for about 20–30 seconds before going to the next step.
4. **Pick a Colony from a Stock Plate:** Open the plate containing your bacterial stock and gently pick up a colony with the loop. Do not gouge the agar while selecting a colony. To prevent contaminants from falling into the plate, keep the lid over the sample like a "clam shell." Withdraw the loop once you have a colony on it, put the plate lid on the culture, and set the plate down on the table.
5. **Open the Sterile Broth Tube:** With your non-loop hand, pick up the sterile tube of broth that you'll inoculate. Then, using the little finger of the hand holding your loop, remove the tube's cap and hold the cap with your pinky; do not put the cap down.
6. **Flame the Broth Tube's Opening:** Holding the open tube at a slight angle, quickly pass the mouth of the tube through the flame a few times (don't let broth slosh out of the tube). If you're using an electric incinerator, hold the mouth of the tube near the entrance of the incinerator.
7. **Inoculate the Sterile Broth:** Dip the loop into the sterile broth and gently swirl the loop.
8. **Remove the Loop:** Before removing the loop, tap the loop's face on the inside of the tube to remove the film within the loop. Be persistent. If you flame the loop with the film present, you'll produce aerosols.
9. **Flame the Newly Inoculated Tube's Opening:** Keeping your loop hand still, flame the tube's opening, replace the cap, and return the tube to the rack. Sample caps should be kept loose for incubation, but not so loose that they will easily fall off.
10. **Re-sterilize the Loop:** Before putting the loop down, re-flame the filament to sterilize it.

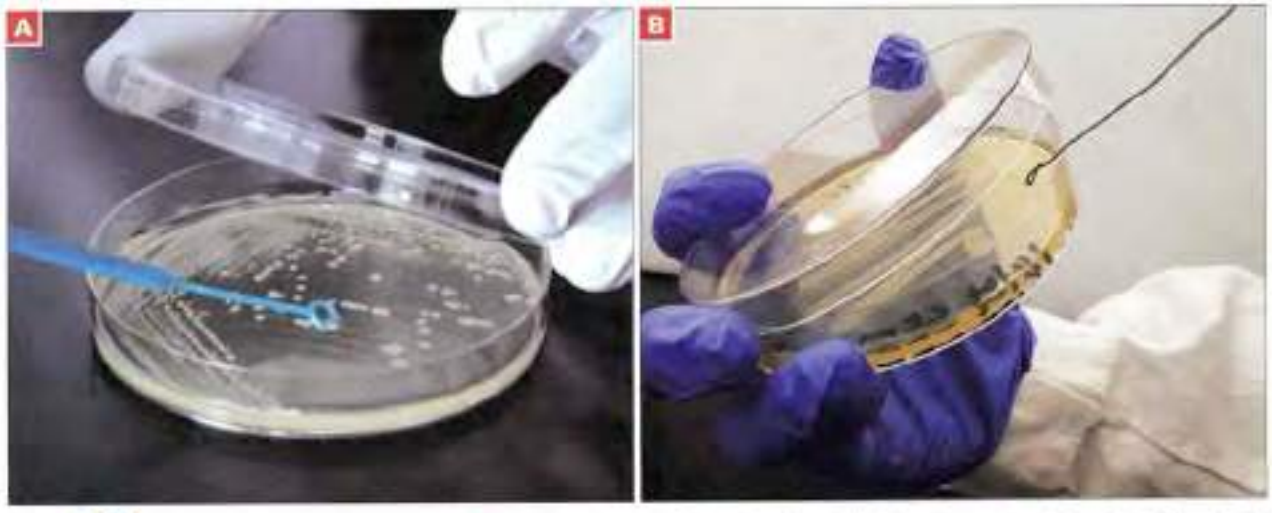
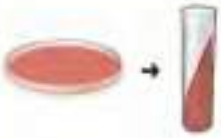


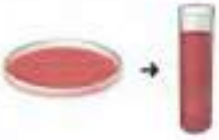
FIGURE **3.9** **"Picking" a Colony from an Agar Plate** • When opening an agar plate, using a "clam shell" angle can prevent contaminants from falling into the sample. (A) You can place the plate on the table to do this; or (B) hold the plate in your hand.



Transferring Culture from a Plate to a Slant

Here you'll pick a colony from an agar plate culture and introduce it to an agar slant. To prevent contamination, remember to hold the plate's lid in a clam shell position over the agar (Fig. 3.9).

1. **Check the Loop:** Ensure your inoculating loop has a fully closed circle at the end, if not, pinch it closed.
2. **Loosen the Cap on the Sterile Slant You'll Inoculate:** Loosen, but don't remove, the cap on the slant you'll inoculate and return the tube to the rack.
3. **Sterilize the Loop:** Hold the inoculating loop in your dominant hand like a pencil and heat the length of the wire filament until each section glows orange. Once the filament is sterilized, do not put it down and don't touch it to any surface. Allow the loop to cool for about 20–30 seconds before going to the next step.
4. **Pick a Colony from a Stock Plate:** Open the plate containing your bacterial stock and gently pick up a colony with the loop. To prevent contaminants from falling into the plate, keep the lid over the sample like a "clam shell." Withdraw the loop once you have a colony on it, put the plate lid on the culture, and set the plate down on the table.
5. **Open the Sterile Slant Tube:** With your non-loop hand, pick up the sterile slant that you'll inoculate. Then, using the little finger of the hand holding your loop, remove the tube's cap. Hold the cap with your pinky; do not put the cap down.
6. **Flame the Tube's Opening:** Holding the open tube at a slight angle, quickly pass the mouth of the tube through the flame a few times. If you're using an electric incinerator, hold the mouth of the tube near the entrance of the incinerator.
7. **Fishtail Inoculation of the Sterile Slant:** Starting at the bottom of the slant, gently drag the inoculating loop across the slant surface in a zigzag pattern until you reach the top of the slant (Fig 3.7). ***Note:*** If you use too much pressure, you'll gouge the agar.
8. **Remove the Loop:** Carefully remove the loop. Avoid catching it on the lip of the tube.
9. **Flame the Newly Inoculated Tube's Opening:** Keeping your loop hand still, flame the tube's opening, replace the cap, and return the tube to the rack. Sample caps should be kept loose for incubation, but not so loose that they will easily fall off.
10. **Re-sterilize the Loop:** Before putting the loop down, re-flame the filament to sterilize it.



Transferring Culture from a Plate to a Deep

This time you'll use a needle instead of a loop to pick a colony from an agar plate culture and introduce the inoculum into an agar deep.

1. **Check the Needle:** Ensure your inoculating needle is reasonably straight, if it's not, straighten the wire. ***Caution!*** Be gentle! The wire easily pulls out of the handle.
2. **Loosen the Cap on the Sterile Deep You'll Inoculate:** Loosen, but don't remove, the cap on the agar deep you'll inoculate and return the tube to the rack.
3. **Sterilize the Needle:** Hold the inoculating needle in your dominant hand like a pencil and heat the length of the wire filament until each section glows orange. Once the filament is sterilized, do not put it down and don't touch it to any surface. Allow the loop to cool for about 20–30 seconds before going to the next step.
4. **Pick a Colony from a Stock Plate:** Open the plate containing your bacterial stock and gently pick up a colony with the loop. To prevent contaminants from falling into the plate, keep the lid over the sample like a "clam shell." Withdraw the needle once you have a colony on it, put the plate lid on the culture, and set the plate down on the table.
5. **Open the Agar Deep Tube:** With your non-needle hand, pick up the sterile deep that you'll inoculate. Then, using the little finger of the hand holding your needle, remove the tube's cap. Hold the cap with your pinky; do not put the cap down.
6. **Flame the Tube's Opening:** Holding the open tube at a slight angle, quickly pass the mouth of the tube through the flame a few times. If you're using an electric incinerator, hold the mouth of the tube near the entrance of the incinerator.
7. **Stab Inoculate the Sterile Agar Deep:** In as straight a line as possible, slowly stab the middle of the agar deep until the needle is as close to the bottom of the agar as possible without having the handle touch the agar (refer to Fig 3.8).
8. **Remove the Needle:** Carefully remove the needle. Avoid catching it on the lip of the tube.
9. **Flame the Newly Inoculated Tube's Opening:** Keeping your needle hand still, flame the tube's opening, replace the cap, and return the tube to the rack. Sample caps should be kept loose for incubation, but not so loose that they will easily fall off.
10. **Re-sterilize the Needle:** Before putting the needle down, re-flame the filament to sterilize it.

Clinical Applications

Health care-acquired infections (HAIs), also known as **nosocomial infections**, are infections that develop as a medical intervention complication. The U.S. Centers for Disease Control and Prevention estimates that in American hospitals alone, there are about 1.7 million HAIs and roughly 99,000 HAI associated deaths each year. One way to prevent these infections is to practice proper aseptic methods. Anytime a health-care provider collects a patient sample (e.g., tissue biopsy, blood, etc.), administers an injected drug, dresses a surgical wound, delivers a baby, inserts a catheter or IV, or performs any sort of invasive procedure, they apply aseptic methods to keep patients safe. Furthermore, since patient samples often contain BSL-2 agents, health-care providers must know how to safely handle such pathogens.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Bunsen burner
- ❑ Inoculating loop
- ❑ Inoculating needle
- ❑ Materials for labeling samples (e.g., marking pen, tape, etc.)
- ❑ Vortex mixer (optional)
- ❑ Nutrient broth culture of *Staphylococcus epidermidis* (or another BSL-1 bacterium) (Part B)
- ❑ Nutrient agar slant culture of *Staphylococcus epidermidis* (or another BSL-1 bacterium) (Part C)
- ❑ Nutrient agar streak plate of *Staphylococcus epidermidis* (or another BSL-1 bacterium) (Part D)
- ❑ Sterile nutrient broth (NB) tubes, 4 per lab group (1 broth tube is used in each exercise)
- ❑ Sterile nutrient agar (NA) slants, 3 per lab group (1 slant tube is used in Parts A, B, and D—a slant is not used in Part C)

PROCEDURE

Your instructor may or may not have you perform all parts (A–D) of the exercise (Table 3.1). Pay attention to their instructions and proceed in the order they direct you.

TABLE **3.1** Inoculations for Parts A–D •

Procedure Part	Source	Destination	Inoculating Tool
A (simulation)	Sterile broth sample	Sterile broth	Loop
		Sterile slant	Loop
B	Broth sample	Sterile broth	Loop
		Sterile slant	Loop
C	Slant sample	Sterile broth	Loop
		Sterile deep	Needle
D	Plate sample	Sterile broth	Loop
		Sterile slant	Loop
		Sterile deep	Needle

PART A: Practicing Aseptic Methods with a Sterile "Stock" Sample

For this part of the exercise, you'll simulate inoculating a broth and a slant. During the next lab session—after potential contaminants have had a chance to grow—you'll check your samples to see if you managed to keep them sterile.

1. Clear your workspace as much as possible.
2. Put on your safety gear (lab coat, safety glasses, and gloves).
3. If using a Bunsen burner, light it and adjust the flame so a small blue cone is at the center (Fig. 3.10). You do not want a large orange flame. If using an incinerator, turn it on.
4. Obtain two tubes of sterile broth and one sterile slant.
5. Label each of your three tubes with your name, the date, and the name of the medium (e.g., nutrient broth or nutrient agar, NB and NA for short). Normally you'd also add the bacterium's name to each label, but we won't be using any bacteria for this practice. Instead, you're going to see if you can keep your samples sterile. As such, label one of the sterile broth tubes "simulated stock" and label each of your other two tubes "simulated inoculation."

6 Use the "simulated stock" to inoculate your broth and slant tubes. To become comfortable with your aseptic methods, inoculate the sample broth three separate times. Do the same for the slant.

7 When you're done, put all three of your labeled tubes wherever your instructor directs you to put them for incubation. Samples are typically incubated overnight at 35–37°C.

→ **Next Lab Session**

1 Remove your "sterile" practice tubes from the incubators and examine them for growth. Record your observations and answer the data sheet questions at the end of this lab.

2 Dispose of your tubes as your instructor directs.

FIGURE **3.10** **Bunsen Burner Flame** • When properly adjusted, a Bunsen burner produces a two-cone flame. The inner cone (red arrow) is the hottest and is where the inoculating tool wire is inserted for sterilization. The outer cone (white arrow) is used to heat the mouths of open glassware items.

PART B: Transferring Bacteria from a Broth Culture to Non-plated Media

For this part of the exercise, you'll transfer bacteria from a stock bacterial broth into fresh sterile broth and onto a sterile agar slant. You'll check your results the next lab session, after the bacteria have had a chance to grow.

1 Clear your workspace as much as possible.

2 Put on your lab coat, eye protection, and gloves.

3 Obtain a stock broth bacterial culture. Your instructor may use *Staphylococcus epidermidis* or some other bacterium.

4 Obtain a tube of sterile broth and a sterile agar slant. Label each tube with your name, the date, the name of the bacterium you will grow, and the name of the medium (e.g., nutrient broth or nutrient agar, NB and NA for short).

5 To inoculate the broth media, follow the directions for transferring a culture from a broth to another broth (p. 53–55).

6 To inoculate the agar slant, follow the directions for transferring culture from a broth to an agar slant (p. 56).

7 After you've completed both transfers, put your newly inoculated samples wherever your instructor directs you to put them for incubation. Samples are usually incubated at 35–37°C overnight. Save or dispose of the original culture as your instructor directs.

→ **Next Lab Session**

1 Remove your cultures from the incubators and examine them for growth. Record your observations and answer the data sheet questions at the end of this lab.

2 Dispose of your samples as your instructor directs.

PART C: Transferring Bacteria from a Slant Culture to Non-plated Media

In this part of the exercise, you'll transfer bacteria from a stock bacterial slant into sterile broth and onto a sterile agar deep. You'll check your results the next lab session, after the bacteria have had a chance to grow.

1 Clear your workspace as much as possible.

2 Put on your lab coat, eye protection, and gloves.

3. Obtain a stock slant bacterial culture. Your instructor may use *Staphylococcus epidermidis* or some other bacterium.
4. Obtain a tube of sterile broth media and a sterile agar deep. Label each tube with your name, the date, the name of the bacterium you will grow, and the name of the medium (e.g., nutrient broth or nutrient agar, NB and NA for short).
5. To inoculate the broth media, follow the directions for transferring a culture from a slant to a broth (p. 57).
6. To inoculate the agar deep, follow the directions for transferring culture from a slant to an agar deep (p. 58).
7. After you've completed both transfers, put your newly inoculated samples wherever your instructor directs you to put them for incubation. Samples are usually incubated at 35–37°C overnight. Save or dispose of the original cultures as your instructor directs.

→ **Next Lab Session**

1. Remove your cultures from the incubator and examine them for growth. Record your observations and answer the data sheet questions at the end of this lab.
2. Dispose of your samples as your instructor directs.

PART D: Transferring Bacteria from a Plated Culture to Non-plated Media

In this part of the exercise, you'll transfer bacteria from a stock bacterial plate into fresh sterile broth, to a sterile agar slant, and into a sterile agar deep. You'll check your results the next lab session, after the bacteria have had a chance to grow.

1. Put on your lab coat, eye protection, and gloves.
2. Obtain a stock plate of bacteria. Your instructor may use *Staphylococcus epidermidis* or some other bacterium.
3. Obtain a tube of sterile broth, a sterile agar slant, and a sterile agar deep. Label each tube with your name, the date, the name of the bacterium you will grow, and the name of the medium (e.g., nutrient broth or nutrient agar, NB and NA for short).
4. To inoculate the broth media, follow the directions for transferring a culture from a plate to a broth (p. 59).
5. To inoculate the agar slant, follow the directions for transferring culture from a plate to a slant (p. 60).
6. To inoculate the agar deep, follow the directions for transferring culture from a plate to a deep (p. 61).
7. After you've completed all three transfers, put your samples wherever your instructor directs you to put them for incubation. Samples are usually incubated at 35–37°C overnight. Save or dispose of the original cultures as your instructor directs.

→ **Next Lab Session**

1. Remove your cultures from the incubator and examine them for growth. Record your observations and answer the data sheet questions at the end of this lab.
2. Dispose of your samples as your instructor directs.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 3

DATA SHEET

Common Aseptic Transfer and Inoculation Methods

During the Lab

1 Describe the appearance of growth on/in each **solid** medium for the lab exercises you performed.

Part A	Part B	Part C	Part D
Slant:	Slant:	Deep:	Slant: Deep:

The degree of cloudiness (turbidity) is often used to qualitatively describe a bacterium's growth in a liquid medium. Using the following scoring scale, note the turbidity of your **broth** samples for each exercise you performed:

+++ for "very turbid"

++ for "somewhat turbid"

\+ for "barely turbid"

0 for "not turbid"

Part A	Part B	Part C	Part D

2 Name at least three ways contaminants could enter a sample in Part A.

3 Considering the cultures used to inoculate each medium in this exercise, how many different microbial types should you expect to see on/in each medium? Explain your answer.

Lab Related Questions

1 Which of the following is (are) required for proper aseptic transfer of a bacterial specimen? (Select all that apply.)

a. A sterile work surface.
b. Sterile media.
c. Limited exposure of the sample to the environment.
d. A sterile inoculating tool.
e. Knowledge of the specimen's biosafety level.

2 Which of the following is (are) described as complex media? (Select all that apply.)

a. Blood agar
b. Glucose minimal salts media
c. Nutrient broth
d. Nutrient agar
e. Skim milk agar

3 Some of the following steps should not be used if you want to aseptically transfer a culture **from a plate to a slant**. First, draw a line through the steps you would not use. Next, place the remaining steps in sequential order, with "1" being the earliest step.

_____ Flame the inoculating needle.
_____ Loosen the slant tube's cap and return the tube to the rack.
_____ Open the plate using a "clam shell" position for the lid.
_____ Flame the mouth of the slant tube and secure the cap.
_____ Perform a fishtail inoculation of the slant surface.
_____ Take the cap off the slant and set the cap on the table.
_____ Stab the agar slant with the inoculating tool.
_____ Pick up a colony with the inoculating tool.

4 Name the four things you should list on every specimen label:

- _____
- _____
- _____
- _____

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 3
DATA SHEET
(continued)

5 Which of the following is (are) mismatched? (Select all that apply.)

a. Broth media: inoculated with a needle
b. Slant media: inoculated with a loop
c. Deep media: inoculated with a loop
d. Colony: consists of a single bacterial species
e. Plate: solid media

6 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ For defined media, each ingredient is precisely known and quantifiable.

_____ Agar is a solidifying agent that most pathogens will digest as a nutrient source.

_____ Clinical samples are usually handled as though they may contain BSL-1 infectious agents.

_____ Slant cultures are ideal for growing large volumes of sample.

_____ A mixed culture contains at least two different media.

7 The best media format for bacterial species isolation is:

a. Broth media.
b. Slant media.
c. Semisolid media.
d. Plate media.
e. Agar deeps.

8 A major concern in working with BSL-2 pathogens is the formation of _____, which can lead to infection through inhalation.

Critical Thinking

1 Some students might notice a difference in density (turbidity) of growth in nutrient broth tubes inoculated from nutrient broth versus inoculated from a nutrient agar slant or plate. Suggest some reasons why a difference may occur.

2 Suppose you performed a transfer from a nutrient broth stock to a glucose minimal salts slant and upon checking your sample the next day, you do not see any growth. Assuming you did everything correctly during the transfer, why might the organism have failed to grow?

EXERCISE 4

Streak Plate Isolation Methods

After doing this exercise, you should be able to:

1. Use three streak plate isolation patterns to inoculate plated media.
2. Correctly apply aseptic techniques while handling bacterial cultures and media.
3. State the risks associated with environmental sampling.
4. Describe how bacterial isolation methods are clinically useful.
5. Define the following terms: *colony, colony-forming unit, mixed culture, pure culture,* and *etiological agent.*

Background

A microbial culture containing two or more species is called a **mixed culture**. In contrast, a **pure culture** contains only a single species. Generally, the first step in identifying an organism is to isolate it from a mixed sample. A common **isolation technique** is the **streak plate**.

In the streak plate isolation method, a bacterial sample is streaked onto an agar plate. The streaking process spreads out the sample so individual cells are scattered across the agar surface. If the medium being used supports the microbe's growth, then sufficiently isolated (separated) cells will grow into individual **colonies** that are visible to the naked eye. A colony is a collection of genetically identical cells that originated from a **colony-forming unit** (CFU). In some cases, a CFU may be an individual cell that was seeded onto the plate, but a pair or even a small chain or cluster of cells can also give rise to a colony. Cells from an isolated colony (one that is not touching another colony) can be transferred to a sterile medium to start a pure culture.

A variety of streak plate inoculation patterns exist; the one used depends on the inoculum's source and the microbiologist's preference. Although streak patterns range in complexity, all are designed to separate deposited cells (CFUs) on the agar surface to generate isolated colonies. The quadrant streak pattern and T-streak pattern are useful when dealing with concentrated samples, whereas a simple zigzag (continuous streak) pattern may be useful for low concentration samples.

The Quadrant Streak Plate Isolation Pattern

As the name suggests, a quadrant streak pattern involves dividing the plate you'll inoculate into quadrants (four sections). You can think of it as a process that dilutes the sample on a solid surface.

Using a sterile loop, the source sample is inoculated into the first section. From there, the sample is spread to the other three sections; we do not add any more sample. To sequentially dilute the sample, the inoculating loop is flame sterilized between each section. The hope is that in at least one section (usually the last section, where the sample is most diluted), the cells will be spread far enough apart that they will grow into isolated colonies (Fig. 4.1). Before you start working, always declutter your work space and put on your safety gear (lab coat, safety glasses, and gloves).

1. **Collect the Sample onto a Sterile Loop:** Your stock sample might be a liquid or it may be growing on solid agar media (i.e., a plate or slant). No matter what the source sample's nature, you'll need to get some of it on a sterile loop. Refer to Exercise 3, page 59, for specific directions on obtaining a sample from a broth sample or a sample growing on agar medium.

FIGURE **4.1** **Quadrant Streak Plate of *Serratia marcescens*** • Note how the growth density decreases as we progress clockwise around the plate from Quadrant 1 to 4. On this plate, some isolation is achieved in the second quadrant, but the microbiologist would not know that at the time of streaking, so all four quadrants are streaked in the hope that isolation will occur in at least one of them.

2. **Positioning the Plate for Inoculation:** Hold your sample-loaded loop in your dominant hand like a pencil. With your free hand, you'll position the plate for inoculation. You have two options for this:
   - **Option 1:** Leave the sterile agar plate on the table and using your free hand, slightly lift the lid into a "clam shell" configuration. In this position, the lid shields the agar surface from airborne contamination (Fig 4.2A).
   - **Option 2:** Hold the plate in your free hand and slightly open it so the lid and plate have a "clam shell" orientation and the lid is in place to serve as a shield against airborne contaminants (Fig. 4.2B).

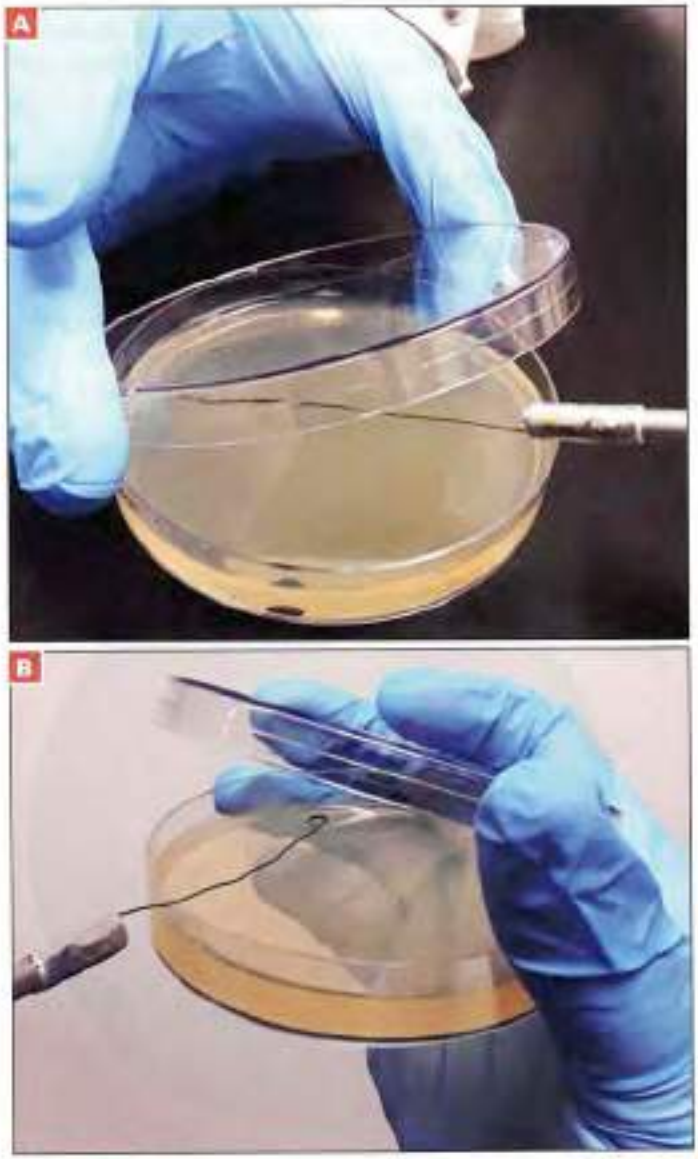


FIGURE **4.2** **Positioning a Plate for Inoculation** • (A) You can place the plate on the table and hold the lid in a "clam shell" configuration to shelter the agar from airborne contamination. (B) If you prefer having the culture closer to eye level, you can use your non-loop hand to hold the plate in a "clam shell" configuration.

3. **Inoculate the First Quadrant:** Starting at the edge of the plate, lightly drag the loop back and forth across the agar surface's first quadrant (Fig. 4.3). Be careful not to cut the agar surface. The loop should contact the agar with slight pressure at an angle like that shown in Figure 4.4.

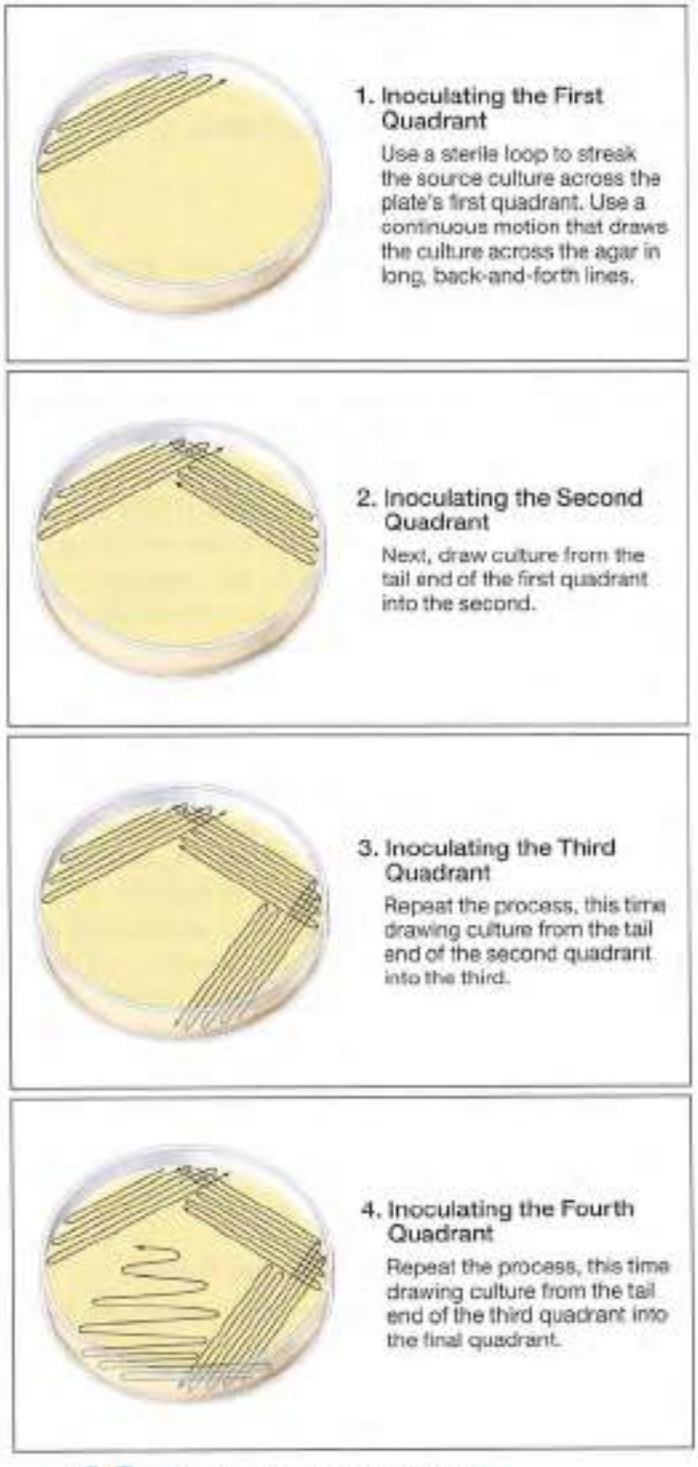

1. Inoculating the First Quadrant
Use a sterile loop to streak the source culture across the plate's first quadrant. Use a continuous motion that draws the culture across the agar in long, back-and-forth lines.
2. Inoculating the Second Quadrant
Next, draw culture from the tail end of the first quadrant into the second.
3. Inoculating the Third Quadrant
Repeat the process, this time drawing culture from the tail end of the second quadrant into the third.
4. Inoculating the Fourth Quadrant
Repeat the process, this time drawing culture from the tail end of the third quadrant into the final quadrant.

FIGURE **4.3** **Performing a Quadrant Streak Pattern** •

4. **Sterilize the Inoculating Loop:** Remove the loop from the plate and replace the lid. Flame sterilize the loop's wire filament using a Bunsen burner's flame or an incinerating machine. Ensure that each section of the wire is heated to a glowing state. Allow the loop to thoroughly cool before proceeding (about 30 seconds). In some cases, your instructor may encourage you to cool the loop by gently touching it to a part of the agar surface that has not been inoculated. You should follow their preferences for this.
5. **Inoculate the Second Quadrant:** The culture for this quadrant will come from the first quadrant. You do not go back into the source culture. Gently drag your sterile, cooled loop across the tail end of the first streak pattern and into the second quadrant as shown in Figure 4.3.
6. **Sterilize the Inoculating Loop:** Sterilize the loop and let it cool.
7. **Inoculate the Third Quadrant:** Repeat the streaking process, this time drag the loop across the tail end of the second quadrant and into the third section (Fig. 4.3).
8. **Sterilize the Inoculating Loop:** Sterilize the loop and let it cool.
9. **Inoculate the Fourth Quadrant:** Repeat the streaking process, but this time, drag the loop across the tail end of the third quadrant and into the fourth (Fig. 4.3).
10. **Sterilize the Loop:** Sterilize the loop and set it down.
11. **Incubate the Plate:** Plates are incubated in an inverted position so condensation won't accumulate on the lid and drip back onto the culture.

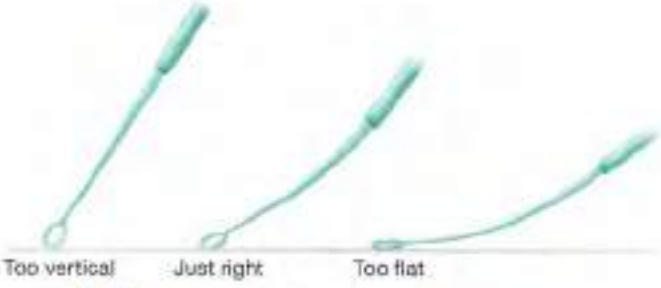


FIGURE **4.4** Proper Loop Angle and Pressure •

The T-streak Plate Isolation Pattern

The T-streak method is a variation on the quadrant streak, but instead of dividing the plate into four sections, three sections are designated (Fig. 4.5). There is no advantage of one method over the other; it comes down to personal preference. Before you start working, always declutter your work space and put on your safety gear (lab coat, safety glasses, and gloves).

1. **Section the Plate:** Use a marking pen to draw a "T" on the base of the plate you'll inoculate (Fig. 4.5).
2. **Collect the Sample onto a Sterile Loop:** Your stock sample might be a liquid or it may be growing on solid agar media (i.e., a plate or slant). No matter

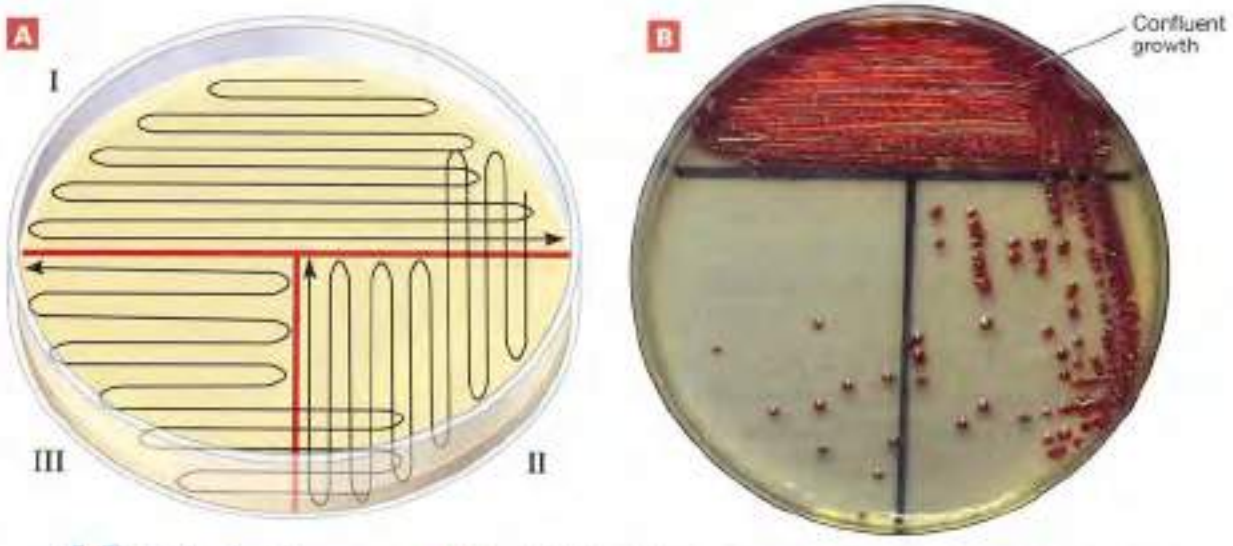


FIGURE **4.5** T-streak of *Serratia marcescens* • (A) This method divides the plate into three sections. The regions are designated by drawing lines (shown in **red**) in the shape of a "T" on the plate's bottom. The first region (I) is the largest (about one-third of the agar's surface) and is streaked with the source sample. After sterilizing the loop, draw it across the tail end of the first section a few times followed by four or five streaks into the second region (II). The final section (III) is similarly inoculated. (B) A T-streak of *Serratia marcescens*.

what the source sample's nature, you'll need to get some of it on a sterile loop. Refer to Exercise 3 Lab Techniques, pages 53–61, for specific directions on obtaining a sample from a broth sample or a sample growing on agar medium.

3. **Positioning the Plate for Inoculation:** Hold your loop-laden sample in your dominant hand like a pencil. With your free hand, you'll position the plate for inoculation. You have two options for this:
   - **Option 1:** Leave the sterile agar plate on the table and using your free hand, slightly lift the lid into a "clam shell" configuration. In this position the lid shields the agar surface from airborne contamination (Fig. 4.2A).
   - **Option 2:** Hold the plate in your free hand and slightly open it so the lid and plate have a "clam shell" orientation and the lid is in place to serve as a shield against airborne contaminants (Fig. 4.2B).
4. **Inoculate the First Section:** Being careful that you don't cut the agar, use the loop to streak your sample across the larger section of the agar—the area above the "T" you drew on the plate.
5. **Sterilize the Inoculating Loop:** Remove the loop from the plate and replace the lid. Flame sterilize the loop's wire filament using a Bunsen burner's flame or an incinerating machine. Ensure that each section of the wire is heated to a glowing state. Allow the loop to thoroughly cool before proceeding (about 30 seconds). In some cases, your instructor may encourage you to cool the loop by gently touching it to a part of the agar surface that has not been inoculated. You should follow their preferences for this.
6. **Inoculate the Second Section:** The culture for this section will come from the first section. You do not go back into the source culture. Gently drag your sterile, cooled loop across the tail end of the first streak pattern and into the second section as shown in Figure 4.5.
7. **Sterilize the Inoculating Loop:** Sterilize the loop and let it cool.
8. **Inoculate the Third Section:** Repeat the streaking process, this time dragging the loop across the tail end of the second section and into the third section (Fig. 4.5).
9. **Sterilize the Loop:** Sterilize the loop and set it down.
10. **Incubate the Plate:** Plates are incubated in an inverted position so condensation won't accumulate on the lid and drip back onto the culture.

Zigzag (Continuous) Plate Inoculation Pattern

This inoculation pattern is usually performed for low concentration samples (e.g., certain environmental samples) or when you know you are dealing with a pure culture and species isolation is not necessary. Before you start working, always declutter your work space and put on your safety gear (lab coat, safety glasses, and gloves).

1. **Obtain Your Sample:** You'll use a sterile swab to collect your sample and inoculate it onto an agar plate. Your sample might be a liquid or it may be growing on solid agar media (i.e., a plate or slant). Alternatively, it may be directly from the environment (e.g., a swipe of a surface).
2. **Position the Plate for Inoculation:** Hold the swab comfortably in your dominant hand and lift the lid of the petri plate with the other. As described in the quadrant and T-streak procedures, use the lid as a shield to protect the agar from airborne contamination.
3. **Inoculate the Agar:** Lightly drag the cotton swab across the agar surface in a zigzag pattern, rolling it as you do so. Be careful not to cut the agar surface (Fig. 4.6).
4. **Replace the Lid and Dispose of the Swab:** Cover the plate, set it down, and then dispose of the swab in a biohazard container.
5. **Incubate the Plate:** Plates are incubated in an inverted position so condensation won't accumulate on the lid and drip back onto the culture.

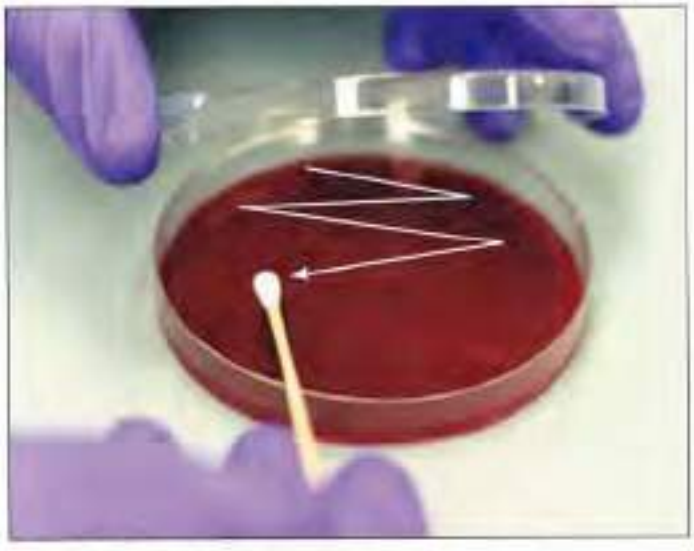


FIGURE **4.6** Zigzag (Continuous) Inoculation with a Swab

A Hybrid Plate Inoculation: Zigzag Inoculation Combined with Streak Plating

This pattern is a hybrid of the zigzag and quadrant plate inoculation patterns. It is usually performed as the initial streak for isolation of two or more bacterial species in a mixed culture with suspected high cell density. Like the quadrant method, the plate is inoculated across four sections, but unlike the quadrant method, the first quadrant is inoculated with a swab. Before you start working, always declutter your work space and put on your safety gear (lab coat, safety glasses, and gloves).

1. **Obtain Your Sample:** You'll use a sterile swab to collect your sample and inoculate it onto an agar plate. Your sample might be a liquid or it may be growing on solid agar media (i.e., a plate or slant). Alternatively, it may be directly from the environment (e.g., a swipe of a surface).
2. **Position the Plate for Inoculation:** Hold the swab comfortably in your dominant hand and lift the lid of the petri plate with the other. As described in the quadrant and T-streak procedures, use the lid as a shield to protect the agar from airborne contamination.
3. **Inoculate the Agar:** Lightly drag the cotton swab across the first quadrant of an agar plate. This replaces the first streaking pattern used in the quadrant isolation pattern.
4. **Replace the Lid and Dispose of the Swab:** Cover the plate, set it down, and then dispose of the swab in a biohazard container.
5. **Complete the Streak Pattern with a Loop:** Further streaking is performed with an inoculating loop as described in Steps 5–10 of the quadrant isolation pattern (see p. 71).
6. **Incubate the Plate:** Plates are incubated in an inverted position so condensation won't accumulate on the lid and drip back onto the culture.

Clinical Applications

If you've ever had your throat swabbed to check for streptococcal pharyngitis (strep throat), then you've been a beneficiary of bacterial isolation methods. The techniques you'll perform in this lab are used every day in clinical labs to isolate and identify an **etiological agent** (causative agent) of a patient's bacterial or fungal infection. Viral pathogens are isolated with different methods. By isolating the causative agent, microbiologists can further characterize the pathogen for antimicrobial drug susceptibility. Drug susceptibility data helps clinicians prescribe a drug regimen that will optimally target the pathogen and improve patient outcomes. Being able to isolate bacteria is also central to food, drug, and water quality testing processes, measures that prevent millions of infections every year.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Bunsen burner or electrical incinerator
- ❑ Inoculating loop
- ❑ Sterile swab (Part C only)
- ❑ Materials for labeling samples (e.g., marking pen, tape, etc.)
- ❑ Vortex mixer (only if using a broth stock culture)
- ❑ Tryptic soy agar (TSA) plates (3; one plate for each part: A, B, and C—fewer plates if not performing all three parts)
- ❑ Tube (1) of sterile distilled water (Part C only)
- ❑ Broth or plated cultures of *Micrococcus luteus* and *Staphylococcus epidermidis* (or some other combination of BSL-1 bacteria) (Parts A and B)

PROCEDURE

In this procedure, you'll learn how to isolate individual organisms from a mixed culture, the first step in producing a pure culture. We'll explore three related streaking techniques, the choice of which one is used depends on the sample's concentration (suspected and/or known bacterial levels). Your instructor may have you do some or all of the following four parts. Pay attention.

PART A: Performing a Quadrant Streak

For this part, you'll use the quadrant streak plate isolation pattern to inoculate a plate of enriched media. During the next lab session—after your bacteria have had a chance to grow—you'll check your plate to see if you achieved colony isolation.

1. Clear your workspace as much as possible.
2. Put on your safety gear (lab coat, safety glasses, and gloves).
3. If using a Bunsen burner, light it and adjust the flame so a small blue cone is at the center. You do not want a large orange flame. If using an incinerator, turn it on.
4. Obtain a stock sample of *Micrococcus luteus* (or another bacterium as your instructor directs). The stock may be growing in a broth or on a plate, it depends on what your instructor wants you to use.

5 Label the plate you will inoculate with your name, the date, and the name of the medium (e.g., tryptic soy agar, TSA for short), and the bacterium's name.

6 Follow the quadrant inoculation procedure described on pages 69–71 to transfer a loopful of the bacterial stock to the agar plate.

7 When you're done, put your plate where your instructor directs you to put it for incubation. Samples are typically incubated overnight at 35–37°C. Remember, plates are incubated in an inverted position so condensation won't accumulate on the lid and drip back onto the culture.

→ Next Lab Session

1 Remove your plate from the incubators and examine it for growth. Record your observations and answer the data sheet questions at the end of this lab.

2 Dispose of your plate as your instructor directs.

PART B: Performing a T-streak

Here you'll perform a T-streak pattern to inoculate an agar plate. You'll check your results the next lab session after the bacteria have had a chance to grow.

1 Clear your workspace as much as possible.

2 Put on your safety gear (lab coat, safety glasses, and gloves).

3 If using a Bunsen burner, light it and adjust the flame so a small blue cone is at the center. You do not want a large orange flame. If using an incinerator, turn it on.

4 Obtain a stock sample of *Staphylococcus epidermidis* (or another bacterium as your instructor directs). The stock may be growing in a broth or on a plate, it depends on what your instructor wants you to use.

5 Label the plate you will inoculate with your name, the date, and the name of the medium (e.g., tryptic soy agar, TSA for short), and the bacterium's name.

6 Follow the T-streak inoculation procedure described on pages 71–72 to transfer a loopful of the bacterial stock to the agar plate.

7 When you're done, put your plate where your instructor directs you to put it for incubation. Samples are typically incubated overnight at 35–37°C. Remember, plates are incubated in an inverted position so condensation won't accumulate on the lid and drip back onto the culture.

→ Next Lab Session

1 Remove your plate from the incubators and examine it for growth. Record your observations and answer the data sheet questions at the end of this lab.

2 Dispose of your plate as your instructor directs.

PART C: Zigzag Inoculation of an Environmental Sample

This part involves sampling the environment with a sterile swab and then performing a zigzag inoculation of the collected sample. *Caution!* Before proceeding, read the following section on environmental sampling.

Environmental Sampling

Environmental sampling is interesting, but it can be hazardous. Never perform environmental sampling unless your instructor expressly tells you to do so, and even then only sample environments your instructor has approved.

You must apply strict caution whenever you're sampling the environment. After all, microbes in our environment infect us all the time, and that's *before* we purposely grow them, a process that concentrates their numbers and increases the infection risk. Because of these risks, many instructors only perform such lab exercises with microbiology majors in upper level courses. Some institutions have entirely forbidden such lab exercises for nonmajors and severely restrict the environments that majors can sample (often body sites, bodily fluids, and facilities that contact such fluids are barred from sampling).

The bottom line is, if you sample the environment, there is a good chance that you'll isolate a dangerous pathogen and concentrate it to an infectious dose level. Moreover, that isolated agent is likely to be fully **virulent** (able to exert its full disease-causing potential) as

opposed to **attenuated** (weakened) like the strains we typically use in teaching lab exercises. As such, if you do perform such lab exercises, you must strictly follow *all* BSL-2 safety measures and only perform such exercises in a lab that is specifically designated as a BSL-2 facility (see the Introduction, pp. 2–4, for specifics on BSL-2 safety practices and facilities).

1. Clear your workspace as much as possible.
2. Put on your lab coat, eye protection, and gloves.
3. Label the plate you'll inoculate with your name, the date, and the name of the medium (e.g., tryptic soy agar, TSA for short), and the nature of the sample (e.g., swab of lab door handle).
4. Obtain a tube of sterile water and a sterile cotton swab. Dip the swab in sterile water. Before removing the swab, remove excess water by pressing the swab against the inside of the tube of sterile water. (For more details, refer to the environmental sampling section of Appendix B, p. 475).
5. Use the damp swab to swipe an environmental surface. Rotate the swab as you sample the surface.
6. Follow the zigzag inoculation procedure described on page 72 to transfer the environmental sample to an agar plate. Dispose of your swab in an approved biological hazard container.
7. When you're done, put your plate where your instructor directs you to put it for incubation. Samples are typically incubated overnight at 35–37°C. Remember, plates are incubated in an inverted position so condensation won't accumulate on the lid and drip back onto the culture.

2

→ Next Lab Session

1. Remove your plate from the incubator and examine it for growth. Record your observations and answer the data sheet questions at the end of this lab.
2. Dispose of your plate as your instructor directs.

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 4

DATA SHEET

Streak Plate Isolation Methods

During the Lab

1 Answer the following questions for each part you completed.

Part A (Quadrant Streak)

Describe the growth on your plate. (If none, explain specifically why you may not have growth. You must be more specific than just writing "I did it wrong.")

Did you achieve colony isolation? If so, in what quadrant(s)?

What would you change for next time?

Part B (T-Streak)

Describe the growth on your plate. (If none, explain specifically why you may not have growth. You must be more specific than just writing "I did it wrong.")

Did you achieve colony isolation? If so, in what quadrant(s)?

What would you change for next time?

Part C
(Zigzag Environmental Streak)

Describe the growth on your plate. (If none, explain specifically why you may not have growth. You must be more specific than just writing "I did it wrong.")

Did you achieve colony isolation?

How many different types of colonies did you detect?

What would you change for next time?

2 Considering the stock cultures used for Parts A and B, how many different microbial types should you expect to see on the plates you inoculated?

Lab Related Questions

1 Which of the following applies to the quadrant streak isolation pattern? (Select all that apply.)

a. You should use an inoculating needle.
b. You will enter the stock samples 4 times, once for each quadrant inoculated.
c. You should use an inoculating loop.
d. The plated media is sterilized after inoculation.
e. A swab is used to start the inoculation, and then you use an inoculating loop.
f. The stock sample used must be a pure culture.
g. This method can be used to obtain a pure culture.

2 Which of the following applies to the zigzag isolation pattern? (Select all that apply.)

a. It can be used if you have a pure culture.
b. It is useful for samples with a low bacterial concentration.
c. It is performed with a sterile swab.
d. It can only be used if the stock sample is a broth culture.
e. It involves a single continuous motion for inoculation.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 4
DATA SHEET
(continued)

3 Assume a T-streak is performed and no colony isolation is observed. What could have led to this scenario? (Select all that apply.)

a. Too much bacterial culture was used to inoculate the plate.
b. The loop was not sterilized between plate sections.
c. The sample was contaminated.
d. The streaks did not overlap between sections.
e. The sample was incubated too long.

4 Describe two things that add to the hazard of environmental sampling.

_____

_____

_____

_____

5 In teaching labs we often use pathogens that are weakened and therefore less likely to cause disease. Such microbes are described as _____.

6 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ A pure culture is derived from a single colony.

_____ One CFU will give rise to multiple colonies.

_____ A CFU is always a single cell.

_____ The quadrant streak plate is essentially a dilution of a sample on a plate.

_____ Viral pathogens can be isolated using the techniques described in this lab exercise.

7 A pathogen that can exert its full disease-causing effects is said to be _____.

8 The best definition of an etiological agent is:

a. the causative agent of an infection.
b. a drug resistant strain of bacteria.
c. a pure isolate of a pathogen.
d. a contaminant of a pure culture.
e. the way an infection is passed from one person to another.

Critical Thinking

1 How can colony sheen, shape, and coloration help you know if you have a pure culture or not?

2 Assume a T-streak is performed and colony isolation is observed in the first section of the plate, but no growth occurs in the other plate sections. What most likely led to this scenario?

EXERCISE 5

Spread Plate Isolation Method

After doing this exercise, you should be able to:

1. Properly perform a spread plate inoculation.
2. State the goals of the spread plate method.
3. Correctly apply aseptic techniques while handling bacterial cultures and media.
4. Describe when a spread plate method is appropriate to use.

Background

In the spread plate method, we add a diluted microbial sample to an agar plate and uniformly spread the sample across the media's surface with a sterile spreader, often a glass rod. Provided the sample is properly diluted, colony forming units (CFUs) will be deposited far enough apart on the agar surface to grow into individual colonies.

Not only is the spread plate method useful for colony isolation, it's also used for enumerating how many live bacteria are in a sample. For enumeration, only plates that grow between 25–250 colonies are considered valid samples. Plates that grow over 250 colonies are labeled as TNTC (too numerous to count) and plates that fall below the 25-colony threshold are designated as TFTC (too few to count). The 250-colony limit is the upward cap because good colony isolation is unlikely the closer we get to the 300 mark. The lower limit is set at 25 to limit the impact that potential contaminants have on colony counts. Because this method is great for bacterial enumeration, it's often used in food and water quality testing protocols.

Performing the Spread Plate Method

Generally, multiple plates are inoculated with successively diluted samples. Here we provided a description of how one plate would be inoculated, but the process would be repeated for each dilution being analyzed. As a reminder, before you begin any lab exercise involving live microbes, declutter your work space and put on your lab safety gear (lab coat, safety glasses, and gloves).

1. **Label Plate(s):** Label the base of each plate you'll inoculate with your name, the date, the organism name (or the sample source if the specific organism is unknown), and any other relevant information (e.g., dilution information).
2. **Arrange Your Work Space:** To minimize fire risk, arrange the alcohol jar, Bunsen burner, and agar plate as shown in Figure 5.1.
3. **Open the Plate You'll Inoculate:** Lift the plate's lid and use it as a shield to protect the agar surface from airborne contamination.
4. **Add Sample to the Plate:** Use a pipette to deposit the designated inoculum volume onto the agar surface (Fig. 5.2). (Refer to Appendices C and D for how to use pipettes.) When finished, disposable pipettes and pipette tips are discarded into a biohazardous waste container.

To prevent the inoculum from soaking into the agar, the remaining steps should be completed within about 15 seconds of adding the sample.

5. **Sterilize the Spreading Tool:** Dip the glass spreading rod into alcohol and then pass it through the flame to ignite the alcohol (Fig. 5.3). Remove the rod from the flame and allow the alcohol to completely burn off. Do not leave the rod in the flame; the combination of the alcohol and brief flaming are enough to sterilize it. ***Caution!*** **Do not drop flaming alcohol onto the work surface, and especially not back into the alcohol-filled jar. If the alcohol in the jar ignites, place the lid over it to smother the flame.**

FIGURE **5.1 Spread Plate Set-up** • The spread plate method requires a Bunsen burner, a screw-cap jar with alcohol, a glass spreading rod, and the plate you'll inoculate. The shown arrangement reduces the chance of accidentally catching the alcohol on fire. Notice the cotton in the jar's bottom to reduce the chance of breaking the glass rod.

6. **Cool the Spreader:** After the alcohol has burned off the spreader, cool the spreader by touching it to the agar surface away from the inoculum. As always, shield the agar surface with the plate's lid to prevent contaminants from entering the plate.
7. **Spread the Inoculum:** Carefully rotate the plate so the rod spreads the sample over the full surface of the media, including the edges (Fig. 5.4A). Alternatively, place the plate on an inoculating turn table and spread the inoculum (Fig. 5.4B).
8. **Finishing the Inoculation:** Remove the rod from the plate, replace the plate's lid, and return the rod to the alcohol.

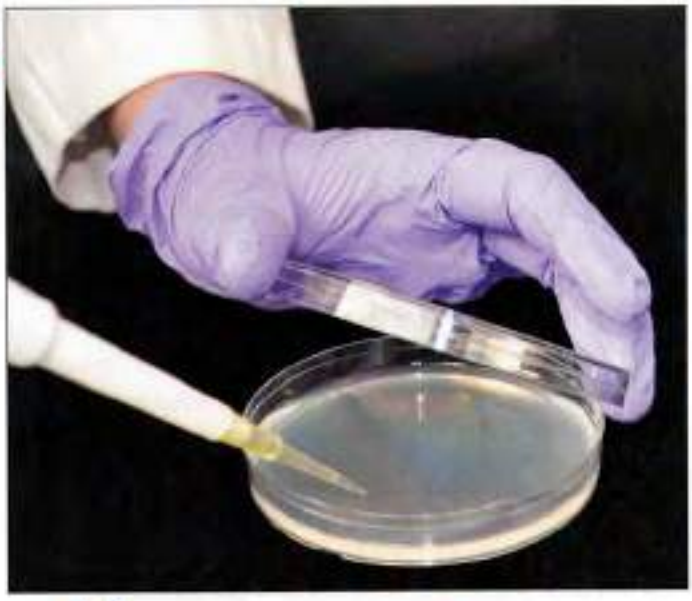


FIGURE **5.2** **Adding the Inoculum to the Plate** • Use a pipette to deposit the inoculum near one side of the agar surface.

FIGURE **5.3** **Flaming the Glass Rod** • Dip the glass spreader into some alcohol and then flame the spreader to burn off the alcohol.

FIGURE **5.4** **Spreading the Inoculum** • (A) Carefully rotate the plate so the rod spreads the sample over the full agar surface, including the edges. (B) An inoculating turntable makes it easy to rotate the plate during the spread plate method.

Clinical Applications

USAID estimates that every day about 1,900 children die of diarrheal diseases linked to poor sanitation and water quality. Effective and widespread water sanitation is one of the hallmarks of an industrialized nation, and it's a process that heavily relies on microbiologists monitoring our water supply. One way this is done is with the spread plate method; a technique that helps microbiologists enumerate the number of live bacteria in a sample. If the level of pathogens exceeds a safe level, then boil water advisories are instated until water quality is restored. The spread plate method is also used to detect and enumerate potential biological contaminants in foods and medications.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Bunsen burner
- ❑ Inoculating loop
- ❑ Materials for labeling samples (e.g., marking pen, tape, etc.)
- ❑ Vortex mixer (optional)
- ❑ Sterile plastic transfer pipettes (6)
- ❑ Glass spreading rod (or 4 sterile disposable spreaders)
- ❑ If using a non-disposable glass spreader, you'll also need a glass, screw-cap jar with cotton at the bottom and enough isopropyl alcohol to only cover the spreading part of the glass rod.
- ❑ Capped microtubes each containing 1 mL of sterile water (4)
- ❑ Empty sterile microtube (1)
- ❑ Nutrient agar (NA) plates (4)
- ❑ Nutrient broth culture of *Staphylococcus marcescens* (or another BSL-1 bacterium)
- ❑ Nutrient broth culture of *Escherichia coli* (or another BSL-1 bacterium)

PROCEDURE

Here you'll perform a spread plate inoculation as an isolation procedure, but it's also commonly used to quantify the number of live bacteria in a liquid sample. Before you begin, clear your workspace as much as possible and put on your safety gear (lab coat, safety glasses, and gloves).

1. **Situate Your Workspace:** Obtain your required materials and situate your workspace so the alcohol-filled jar is not near the Bunsen burner.
2. **Use the Empty Sterile Microtube to Setup Your Culture Stock:** Label the empty microtube "non-dilute stock." Use a sterile pipette to add a few drops of *E. coli* culture to the sterile tube. Then use a second pipette (or a new sterile pipette tip) to add *S. marcescens* culture to the same microtube. Cap the tube and mix the sample with a vortex mixer or by gently drawing and dispensing the mixture in and out of the tube a couple of times with the pipette. Do not spray the mixture! Properly dispose of pipettes or pipette tips into the appropriate biohazardous waste container.
3. **Label the Four Microtubes of Water:** Label the four microtubes containing sterile water "A," "B," "C," and "D."
4. **Label Your Plates:** Label the four nutrient agar plates "A," "B," "C," and "D."
5. **Add Bacteria to Tube A:** Use a sterile inoculating loop to transfer a loopful of the "non-dilute stock" you prepared in Step 2 above into Tube A and mix the contents with the loop or a vortex mixer. If using a vortex mixer, cap the tube before mixing.
6. **Add Bacteria to Tube B:** Using a flame sterilized and cooled loop, transfer a loopful of the mixture in Tube A to Tube B and mix well with the loop or a vortex mixer.
7. **Add Bacteria to Tube C:** Using a flame sterilized and cooled loop, transfer a loopful of the mixture in Tube B to Tube C and mix well with the loop or a vortex mixer.
8. **Add Bacteria to Tube D:** Using a flame sterilized and cooled loop, transfer a loopful of the mixture in Tube C to Tube D and mix well with the loop or a vortex mixer.
9. **Add Bacteria to Plate A:** Use a sterile pipette to add a couple of drops of culture from Tube A to Plate A. Properly dispose of the pipette/pipette tip into a biohazardous waste container. Spread the inoculum with a glass rod. (Follow the steps for performing the spread plate method that were presented on pp. 81–82.)
10. **Inoculate the Remaining Plates:** Repeat Step 9 for Tubes B, C, and D and Plates B, C, and D, respectively. Do your best to transfer the same volume to each plate.
11. **Incubate Your Plates:** Plates are incubated overnight at 35–37°C in an inverted position so condensation won't accumulate on the lid and drip back onto the culture.
12. **Clean up:** Place Tubes A, B, C, D, and your "non-diluted stock" where your instructor directs (typically in a non-sharps biological waste container for autoclaving).

→ Next Lab Session

1. Retrieve your plates from the incubator and examine them for growth. *S. marcescens* produces reddish-orange colonies and *E. coli* produces buff-colored colonies.
2. Record your observations and answer the data sheet questions at the end of this lab. Dispose of your plates as your instructor directs.

2

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 5

DATA SHEET

Spread Plate Isolation Method

During the Lab

1 Record your observations in the table provided.

Organism	Which plates had isolated colonies (A, B, C, and/or D)?	Comments
E. coli		
S. marcescens		

2 When performing the lab, what would be the primary consequence of failing to evenly spread the inoculum over the agar surface?

Lab Related Questions

1 Which of the following is (are) used in the spread plate method? (Select all that apply.)

a. Inoculating needle
b. Nutrient broth
c. Nutrient agar
d. Agar deeps
e. Bunsen burner
f. Glass spreader
g. Alcohol

2 Some of the following steps should *not* be used if you want to aseptically transfer a culture to perform the spread plate method. First, draw a line through the steps you would *not* use. Next, place the remaining steps in sequential order, with "1" being the earliest step.

_____ Select a colony with a sterile loop and inoculate it onto the agar plate.

_____ Add inoculum to a plate with a swab.

_____ Spread the liquid inoculum on the agar surface.

_____ Mix the stock inoculum.

_____ Use a sterile pipette to add the inoculum to an agar plate.

_____ Incubate the plate in an inverted position.

3 Assume you use the spread plate method to grow a series of bacterial samples. Following incubation, you make the following observations:

- Plate A (non-diluted stock A): 500 red colonies; 10 green colonies; 5 white colonies.
- Plate B (10-fold dilution of stock A): 50 red colonies; 1 green colony; 0 white colonies.

Based on this information, if you were to inoculate a third plate (Plate C), which was inoculated using a 10-fold dilution of the stock that was used to inoculate Plate B, what do you predict you'd see following incubation?

a. Plate C would look like Plate A.

b. Plate C would look like Plate B.

c. Plate C would have about 20 red colonies, 1–5 green colonies, and 0 white colonies.

d. Plate C would have 5–10 red colonies and no green or white colonies.

4 Why must solid media be used for colony isolation?

Critical Thinking

1 Suppose you have two organisms in a mixture and Organism A is 1,000 times more abundant than Organism B. Will you (without counting on good luck!) be able to isolate Organism B using the spread plate method? Explain your answer.

2 To get isolated colonies on a plate inoculated using the spread plate method, there can only be about 300 cells in the inoculum. What will happen if the inoculum's cell density significantly exceeds this number?

EXERCISE 6

Colony Morphology

After doing this exercise, you should be able to:

1. Describe bacterial colony morphological features.
2. Name things that can affect colony morphological features.
3. Measure and describe bacterial colony sizes.
4. Discuss why noting bacterial colony morphology is useful in microbiology.
5. Safely handle bacterial cultures and properly dispose of such cultures.

Background

Previously we reviewed inoculation techniques for growing isolated bacterial colonies on solid media. Here we'll review the features we note when observing isolated bacterial colonies. Carefully documenting bacterial colony appearance can help you determine what species are in a sample. Bacterial colony diversity is an important indicator of whether you're dealing with a pure or mixed culture. If the sample is pure (contains only one bacterial species), then every colony on the plate should have the same general **colony morphology**, observable physical characteristics such as color, shape, texture, colony margins, and other features we'll review herein (Fig. 6.1). In contrast, a mixed culture will yield diverse colonies (Fig. 6.2).

Examples of Colony Morphological Features

Round, Shiny, Convex Colonies

These buff-colored colonies are a 48 hour culture of *Providencia stuartii* on nutrient agar. *P. stuartii* is a common isolate from urine samples of catheterized patients. It's highly resistant to antibiotics.

The colonies of the soil and water bacterium *Chromobacterium violaceum* grown on sheep blood agar are purple (hence "violaceum"). Figure 6.4 shows how nutrient availability affects *C. violaceum*'s pigment production.

Round, Dull, Convex Colonies

Dry, buff-colored *Corynebacterium xerosis* colonies on sheep blood agar photographed from the side. *C. xerosis* is an opportunistic pathogen.

This is a close-up of the same *C. xerosis* colonies pictured to the left, but here they have been photographed from above.

FIGURE **6.1** Bacteria Exhibit Diverse Colony Morphology ■

An organism's genetic makeup combined with environmental factors (e.g., nutrient availability, temperature, and incubation time) influence colony morphology (Figs. 6.3 and 6.4). For example, *Serratia marcescens* makes a red pigment called **prodigiosin**. However, the enzyme that helps make this pigment tends to work best below body temperature. As such, we are unlikely to see the red pigmentation unless the bacteria are grown below

Throat Cultures Grown on Sheep Blood Agar
Magnified View of Region
β-hemolysis around a white colony
Whole Plate View
Five unique colonies are evident, indicating a mixed culture

FIGURE **6.2** **Soil and Throat Cultures Demonstrate Microbial Diversity** • Colony features help us identify bacterial species. For example, throat cultures are often inoculated onto blood agar to detect *Streptococcus pyogenes* (the causative agent of streptococcal pharyngitis, strep throat). This pathogen grows as white colonies that exhibit β-hemolysis (break down red blood cells). For information about hemolytic reactions on blood agar plates, see Exercise 25.

37°C. Colony morphological characteristics can be observed with the naked eye, a hand lens, a stereo (dissecting) microscope, and/or a colony counter (Fig. 6.5). Morphological features worth documenting include: colony size, shape (form), margin (edge), elevation, consistency, surface texture, color, and optical properties (Fig. 6.6). Even members of the same genus can manifest diverse morphological features (Fig. 6.7).

- **Size:** We note the diameter of round colonies. For noncircular colonies, we note the colony length and width. In general, colonies are described as **punctate/punctiform** (pinpoint size colonies that are generally smaller than 1 mm), small (1–2 mm), medium (3–4 mm), or large (greater than 5 mm) (Fig. 6.8).
- **Shape** (form): Colonies may be described as **round** (circular), **irregular**, **rhizoid** (root-like), **filamentous**, or **spindle** shaped (wide in the middle and tapered at the ends) (Figs. 6.9 and 6.10).
- **Margin** (edge): The edges or margins of a colony may be **entire** (smooth, with no irregularities), **undulate** (wavy), **lobate** (lobed), **filamentous** (unbranched strands), or **rhizoid** (root-like branching) (Fig. 6.11).
- **Elevation:** Colonies can appear **flat**, **raised**, **convex**, **pulvinate** (very convex), and **umbonate** (raised in the center) (Fig. 6.12).
- **Optical Properties** (opacity, translucency, color): Opaque colonies limit light transmission (they are not transparent), while translucent colonies allow light to pass through them and therefore appear "clear" or "see-through" (Fig. 6.13).
- **Consistency:** The growth may be **moist**, **mucoid** (sticky like mucus), **butyrous** (buttery), or **dry** (Fig. 6.14).
- **Surface Texture:** Colonies can appear **smooth**, **rough**, **rugose** (wrinkled), **granular**, **shiny**, or **dull** (Fig. 6.14).

Features such as colony shape, margin, surface, texture (shiny or dull), and color are best viewed by observing from above. If your instructor says it is safe to do so, you can observe the colonies with the lid off the culture to get a better idea of how light reflects off the colonies. If your instructor allows it, you may also check texture by touching the growth with an inoculating loop or wooden stick. When finished, flame the loop to sterilize it or dispose of the wooden stick in the appropriate biohazard container.

Colony elevation is best viewed with the plate tilted slightly at eye level. Opacity and translucence are best viewed by placing the plate on a colony counter or holding it (lid on) so it is illuminated from behind (transmitted light). Colony dimensions are best measured from the plate's base rather than through the lid.

When reporting colony morphology, it is important to include the medium and the incubation time and temperature, all of which can affect a colony's appearance.

Factors that Affect Colony Features: Incubation Time

Close-up of *Bacillus subtilis* on sheep blood agar after 24 hours of incubation.

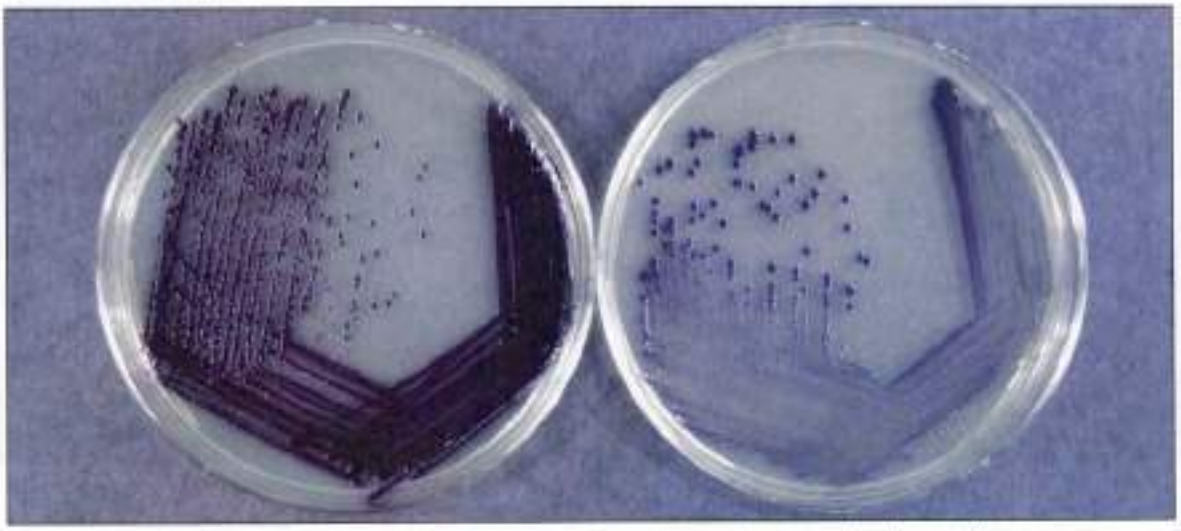
Close-up of *Bacillus subtilis* culture after 48 hours of growth. Note the wormlike extensions.

FIGURE **6.3** Effect of Incubation Time on Colony Morphology •

Factors that Affect Colony Features: Incubation Time, Temperature, and Nutrients

Effect of Time: *Serratia marcescens* grown on nutrient agar for 24 hours (left) and after 48 hours (right). The colonies grew darker over time.

Effect of Temperature: *Serratia marcescens* produces less orange pigment when grown at 37°C (left) than when grown at 25°C (right).



Effect of Nutrients: *Chromobacterium violaceum* produces a much more intense purple pigment when grown on tryptic soy agar (left) than when grown on nutrient agar (right), a less nutritious medium.

FIGURE **6.4** Environmental Factors Can Alter Colony Pigmentation •

FIGURE **6.5** **Colony Counter** • A colony counter provides magnification and transmitted light to help us see subtle differences in colony shape and size. However, colony color and many other features are best determined with reflected light. The grid in the background is a counting aid; each big square is 1 square centimeter.

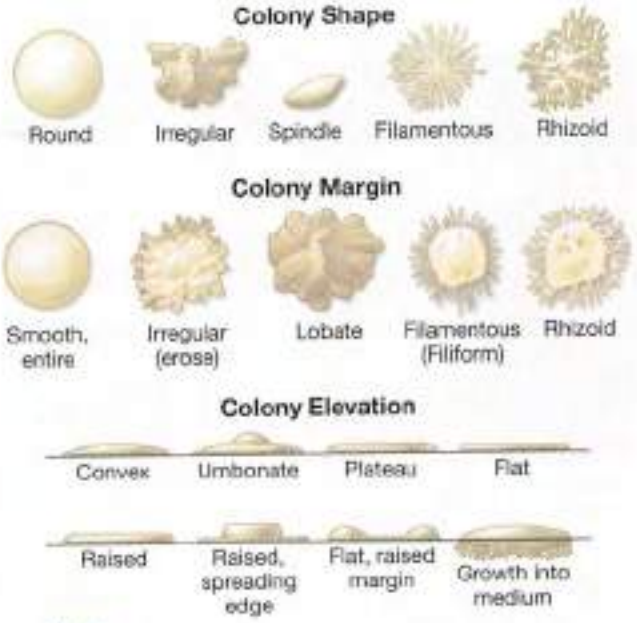


FIGURE **6.6** **Bacterial Colony Morphology Examples** • These terms describe colony morphology. Other descriptions include surface texture, consistency, elevation, color, and optical properties.

Morphological Diversity Among Members of the Same Genus

Bacillus cereus grown on sheep blood agar (SBA) produces distinctively large (up to 7 mm), gray, granular, irregular colonies. They often produce a "mousy" smell. Also note the distinctive extensions of growth along the streak line.

Bacillus anthracis colonies on SBA resemble *B. cereus*, but are usually smaller and adhere to the medium more tenaciously. It must be handled at *least* in a BSL-2 laboratory, and sometimes BSL-3 if the cell density is high enough, so it is unlikely you will be testing its tenacity!

Bacillus mycoides produces distinctive, rapidly spreading, rhizoid (note the branching) colonies. It is a common isolate from soil and is shown here on SBA.

This unknown *Bacillus* isolated as a laboratory contaminant produced a wrinkled, irregular colony with an irregular (wavy) margin on tryptic soy agar.

FIGURE **6.7** **Comparison of Four *Bacillus* Species Colonies** •

Colony Size

Punctiform: These are *Mycobacterium smegmatis* colonies growing on sheep blood agar. This slow-growing relative of *M. tuberculosis* forms punctate colonies that are less than 1 mm in diameter.

Punctiform: *Kocuria rosea* can be isolated from water, dust, and salty foods. Even after growing for 96 hours, colonies are smaller than 0.5 mm in diamter.

FIGURE **6.8** Examples of Punctate Colonies

Colony Shape: Filamentous

These are *Rhizobium leguminosarum* colonies on brain-heart infusion agar. The colonies are about 5 mm in diamter, filamentous, convex, circular, and mucoid with translucent edges. This bacterium aids in nitrogen fixation by forming root nodules in many legumes (*rhiz* = "root").

These are *R. leguminosarum* colonies as viewed through a stereo microscope. The bacteria are growing on brain-heart infusion agar.

Some molds grow as filamentous colonies. This is an unidentified mold mycelium viewed with a stereo microscope.

FIGURE **6.9** Examples of Filamentous Shaped Colonies

Colony Shape: Irregular

This unidentified contaminant grew on tryptic soy agar and was isolated from a laboratory tabletop. In addition to its irregular shape, it has a lobed margin and wrinkled (rugose) surface. Its widest dimension was approximately 5 mm. This photo was taken through a stereo microscope.

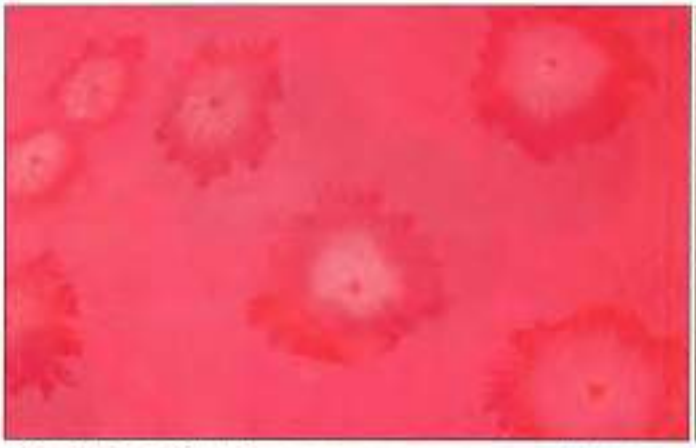
Viewing *Kocuria rosea* colonies with a stereo microscope reveals irregular shaped colonies with a wrinkled surface.

FIGURE **6.10** Examples of Irregularly Shaped Colonies

Common Colony Margins

Smooth Entire Margin: These circular and convex *Rhodococcus rhodochrous* colonies were grown on brain-heart infusion agar for 48 hours. They are about 1 mm in diameter with a smooth margin and a shiny, pink surface (hence "*rhodo*"). *Rhodococcus* species are soil organisms.

Spreading Margin: *Erwinia amylovora* colonies have a raised center with an irregular, spreading margin. These colonies were grown on nutrient agar 48 hours at 25°C and ranged in size from 4 to 6 mm in diamter. *E. amylovora* is a plant pathogen; its name literally means "Erwin (Smith's) starch devourer."



Rhizoid Margin: *Clostridium sporogenesis*, a common soil bacterium, was grown anaerobically on sheep blood agar and viewed via a stereo microscope. Note the irregular, flat, colonies have a raised center and a spreading margin of branched, tangled filaments (like the mythological creature Medusa, who had snakes for hair). Colonies were 2 to 6 mm.

Rhizoid Margin: Fungi (other than yeast) are naturally filamentous and sometimes the filaments are branched, as in this lab contaminant (probably *Aspergillus niger*). It takes magnification to see that the margin is rhizoid and not simply filamentous. The black spheres are asexual spores called conidia.

Lobed Margin: Note the lobed margin and rugose surface of this lab contminant. After 48 hours of incubation at 35°C, this circular colony was about 1 cm in diameter.

Filamentous Margin: This unidentified lab contaminant's colony margin is filamentous. The colony is also circular, convex, shiny, and about 2 mm in diameter.

FIGURE **6.11** Examples of Colony Margins •

Colony Elevation

Flat: This unknown soil isolate is flat only around the edge. The center is slightly concave like a bowl and at the very center is another small, raised ring surrounding another concavity. It's almost like an automobile tire around the wheel and hub. Very unusual! Also note the dull surface.

Umbonate: The colony of this anaerobic lab contaminant on the left is truly umbonate. The one on the right is getting there. Their diameters are about 3 mm.

Umbonate: These are *Enterococcus faecium* colonies grown on tryptic soy agar for 48 hours and photographed via a stereo microscope. The colonies are 1–2 mm in diameter, white, and circular with an entire margin. They are also umbonate—flat except for the central bump. *E. faecium* is found in human and animal feces.

FIGURE **6.12** Examples of Colony Elevations •

Optical Properties

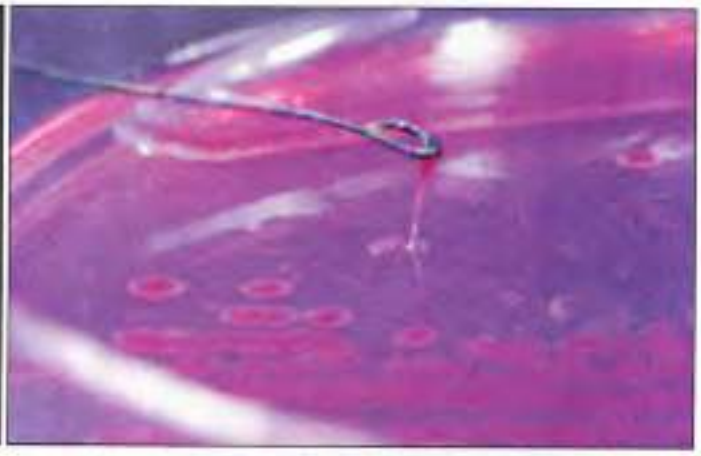
If we only observe reflected light, optical properties can be hard to determine. These colonies were photographed with a stereo microscope using refected light. There are slight differences in size and color, and one is irregular in shape, whereas the others are circular.

When the same colonies from the photograph above are illuminated with transmitted light, it becomes obvious that the medium-sized round colonies are translucent, whereas the others are opaque.

FIGURE **6.13** Viewing Optical Properties with Transmitted Light •

Colony Consistency and Texture

Mucoid Consistency/Shiny Texture: These *Klebsiella pneumoniae* colonies grown on nutrient agar are mucoid, raised, and shiny. While it's a normal inhabitant of the human intestinal tract, it is associated with community-acquired pneumonia and nosocomial urinary tract infections.



Mucoid Consistency/Shiny Texture: *Pseudomonas aeruginosa* is found in soil and water and it easily infects burn wounds. Clinical samples tend to grow as either mucoid colonies with a shiny texture (as shown here), or as "fried-egg" colonies—large, smooth colonies with flat edges and an elevated center.

Butyrous Consistency/Dull Texture: This unidentified 12 mm colony was found on a glycerol yeast extract plate inoculated with a diluted soil sample. Butyrous (butyrum means "buttery") colonies have the consistency of melted butter. This colony is dull in texture as compared to the shiny yellow colony to its right.

Dry Consistency/Rugose Texture: This is *Streptomyces griseus* grown on brain-heart infusion agar. The circular colony has entire margins. At a later stage of development, this bacterium produces yellow reproductive spores. These bacteria have an "earthy" smell. The plate this colony was on made the whole incubator smell like soil!

Dry Consistency/Granular Texture: This is *Streptomyces violaceus* viewed with the stereo microscope after 3 weeks of incubation at 25°C. The colonies are umbonate, dry, and granular. Members of this genus share a growth pattern similar to common fungi—but, they are Gram-positive bacteria and the similarities are only coincidental.

FIGURE **6.14** Diversity of Colony Consistency and Texture •

Clinical Applications

Recognizing different bacterial growth morphologies on agar plates is a useful step in species identification; it's often the first indication that one organism is different from another. Once a pure culture has been confirmed by an appropriate staining procedure (this is not always done), cells can be transferred to a sterile medium, grown, and maintained as a pure culture, which then acts as a source of that microbe for identification or other purposes.

In some cases, bacterial colonies have such a distinct appearance on certain media, that visual properties alone can indicate the presence of a pathogen. For example, *Pseudomonas aeruginosa*, a bacterium that commonly causes wound infections and **otitis externa** (outer ear infections such as swimmer's ear), makes a green-blue pigment called **pyocyanin**. This distinct coloration makes it easy to detect *P. aeruginosa* colonies on certain solid media (Fig. 6.15). Some bacteria exhibit unique growth patterns, such as the swarming growth of *Proteus* species. This swarming ability is thought to help *Proteus* species such as *P. mirabilis* and *P. vulgaris* cause urinary tract infections in catheterized patients (Fig. 6.16).

FIGURE **6.15** **Pyocyanin Pigment** • Here *Pseudomonas aeruginosa* is growing on tryptic soy agar. The characteristic blue-green pigment pyocyanin is evident.

FIGURE **6.16** **Swarming Growth** • Members of the genus *Proteus* will swarm at certain intervals and produce a pattern of concentric rings because of their motility. This photograph demonstrates the swarming behavior of *P. vulgaris* on DNase agar.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Metric ruler
- ❑ Plates inoculated with bacterial samples (e.g., plates from Exercises 4 and/or 5 would work)
- ❑ Colony counter, stereo (dissecting) microscope, and/or hand lens (magnifying glass) (optional)
- ❑ Tryptic soy agar (TSA) or brain-heart infusion agar (BHIA) streak plate cultures of any of the following microbes (optional):
  - ☐ *Bacillus subtilis*
  - ☐ *Corynebacterium xerosis*
  - ☐ *Kocuria rosea*
  - ☐ *Lactobacillus plantorum* or *Lactobacillus acidophilus*
  - ☐ *Micrococcus luteus*
  - ☐ *Mycobacterium smegmatis* (BSL-1 strains are available, i.e., ATCC 27203)
  - ☐ *Pseudomonas aeruginosa* (Strain ATCC 39327 is a BSL-1 strain, other strains may be BSL-2)
  - ☐ *Proteus mirabilis* (BSL-2)

PROCEDURE

Today you will view and describe the growth on plates saved from prior exercises (e.g., Exercises 4 and/or 5) and/or prepared streak plates your instructor provides. Before you begin, put on your safety gear (lab coat, safety glasses, and gloves). *Safety Note:* Many microorganisms are opportunistic pathogens, so handle every plate carefully. Do not open plates cultured from the environment, plates with known BSL-2 organisms on them, or plates containing "fuzzy" growth. The presence of a fuzzy colony suggests fungal growth containing spores that can spread easily and contaminate the laboratory and other cultures. If in doubt about what you can and cannot open, check with your instructor.

1. **Situate Your Workspace:** Obtain your required materials and situate your workspace so it is not cluttered.
2. **Describe Observed Colonies:** Use the terms in Figure 6.6 and in the exercise's background information to describe some representative colonies on the plates you're observing (e.g., plates from Exercises 4, 5, and/or plates provided by your instructor). You may also find Figures 6.7 through 6.14 useful. If you see a distinctive feature that has not been given a name, make up one! Just make it descriptive and easily understood by others. That's what the early microbiologists did to compile the list you have been given.
3. **Measure Observed Colonies:** Use a metric ruler to measure colony sizes (mm scale). Try to find punctate, small, medium, and large colonies. Include colony measurements with your colony descriptions.
4. **Record Your Findings:** Record colony features and measurements in the data sheet at the end of this lab.
5. **Clean up:** Unless you are told to save the samples, discard plates into an appropriate biohazard container.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 6

DATA SHEET

Colony Morphology

During the Lab

1 Use the terms provided in the laboratory exercise background and figures to describe representative colonies on the plates you examined. Draw separate sketches for the colonies as seen from above and in elevation (from the side). Use a colony counter for magnification if necessary. Measure colony diameters, and include them with your descriptions along with incubation times and temperatures, and culture sources.

Organism/Plate	Colony Description	Colony Sketch

2 Based on your observations, which plates contained pure cultures and which (if any) contained mixed cultures?

Lab Related Questions

1 Which of the following does not describe colony elevation?

a. Rugose
b. Raised
c. Flat
d. Pulvinate
e. Concave

2 Provide terms to describe the pictured bacterial colony.

Shape: _____

Margin: _____

Elevation: _____

Texture: _____

3 Based on the following pictured bacteria, how many different species are most likely present? Explain your reasoning.

_____

_____

_____

_____

4 Upon growing bacteria isolated from a burn wound, you observe a greenish-blue bacterial colony on the agar plate. Which of the following is the most likely candidate for the bacterium's identity?

a. *Serratia marcescens*
b. *Pseudomonas aeruginosa*
c. *Streptococcus pyogenes*
d. *Streptomyces griseus*
e. *Kocuria rosea*

5 What feature(s) help *Streptococcus pyogenes* stand out from other bacteria when grown on sheep's blood agar?

_____

6 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ Colony morphology may be affected by incubation temperature.

_____ Bacteria of the same genus may have different colony features.

_____ Colony optical properties are best determined by illuminating a sample with reflected light.

_____ A mixed culture will contain uniform appearing colonies.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 6
DATA SHEET
(continued)

7 *Serratia marcescens* makes a pigment called _____,

which has a _____ coloration.

8 The presence of a fuzzy colony on an agar plate most likely suggests the presence of:

a. A true pathogen.

b. An opportunistic pathogen.

c. *Streptococcus pyogenes*.

d. A bacterial contaminant.

e. A fungus.

Critical Thinking

1 Suppose you observe a bacterial plate that contains numerous colonies and aside from colony size, the bacteria have identical morphological features. Can you safely assume you have a mixed culture? Why or why not?

2 Assume you grew three bacterial samples on brain-heart infusion agar and you obtained the following results:

Plate 1 incubated 37°C for 48 hours: All bright-yellow pigmented colonies.

Plate 2 incubated at 35°C for 24 hours and 37°C for the last 24 hours: All light-yellow pigmented colonies.

Plate 3 incubated at 35°C for 48 hours: All white colonies.

If we assume the cultures were identical and consisted of the same pure culture, then what is the *most likely* explanation for these findings? How could you most easily test your hypothesis?

— Lab Notes —

EXERCISE 7

Growth Patterns on Slants

After doing this exercise, you should be able to:

1. Describe morphological features of bacterial cultures cultivated on agar slants.
2. Discuss the advantages of using agar slants over agar plates.
3. Safely handle bacterial cultures and properly dispose of such cultures.

Background

Agar slants are readily sealed and easily stored in test-tube racks, which means they take up less space than agar plates. These features make slant media a great alternative for cultivating and storing pure stock cultures. That said, slants are not useful for isolating pure cultures, that is best done using plated agar.

Organisms cultivated on slants may display diverse growth characteristics. Many of the organisms you will see in this laboratory exercise have **filiform** growth (dense and opaque with a smooth edge), but you will see differences in other features. Growth on slants can vary in texture (e.g., moist or **friable** [dry, crusty]), optical properties (e.g., **opaque, translucent**), and margin (e.g., **smooth, lobed, spreading/effuse, filamentous, rhizoid** [branched], or **echinulate** [spiny]). Be advised, margins can be difficult to evaluate—and possibly unobservable—if the slant surface was not correctly streaked with an inoculating loop in a defined zigzag (fishtail inoculation) and/or if the organism covers the entire slant with its edge butting up against the glass.

Figure 7.1 illustrates some of these features. Color is also a variable feature of growth on slants. All the organisms in Figure 7.2 are naturally **pigmented** (an obvious difference) and all but one is filiform (the second from the left is effuse). As with growth on plated media, environmental factors such as incubation time and temperature can influence microbial growth patterns. Figure 7.3 illustrates how temperature affects *Serratia marcescens*' pigmentation.

FIGURE **7.1** **Growth Patterns on Slants** • From left to right: filiform (smooth, even growth) and translucent; friable and opaque; effuse and translucent; echinulate and opaque; and rhizoid and opaque.

FIGURE **7.2** **Pigment Production on Slants** • These organisms all naturally produce a pigment. From left to right: *Staphylococcus epidermidis* (white), *Pseudomonas aeruginosa* (green), *Chromobacterium violaceum* (violet), *Serratia marcescens* (red/orange), *Kocuria rosea* (rose), *Micrococcus luteus* (yellow). All but *Pseudomonas*, which is effuse, have an entire (smooth) edge. The waviness you see is due to the inoculation pattern. Bacteria grow where you put them!

FIGURE **7.3** **Influence of Temperature on Pigment Production in *Serratia marcescens*** • *Serratia marcescens* was grown for 48 hours on tryptic soy agar slants at five different temperatures. From left to right: 25°C, 30°C, 33°C, 35°C, and 37°C. A difference of 2°C makes the difference between being pigmented or not!

Clinical Applications

Slants are usually inoculated with pure cultures, so a streak plate has already been done and colony morphology has been described ahead of culturing samples on a slant. Still, growth characteristics on slants can provide useful information when attempting to identify an organism. As you'll see in later lab exercises, many biochemical tests that are central to bacterial identification are slant media.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Slant cultures of the following recommended bacteria:
  - ☐ *Bacillus subtilis*
  - ☐ *Corynebacterium xerosis*
  - ☐ *Kocuria rosea*
  - ☐ *Lactobacillus plantarum* or *Lactobacillus acidophilus*
  - ☐ *Micrococcus luteus*
  - ☐ *Mycobacterium smegmatis* (BSL-1 strains are available—i.e., ATCC 27203)
  - ☐ *Proteus mirabilis* (BSL-2)
- ❑ Uninoculated tube of the slant medium used for the recommended cultures

PROCEDURE

In Exercise 6, you examined colony growth on agar plates. Today you will be looking at the growth patterns of up to seven organisms on prepared agar slants. If your instructor had you save your plates from Exercise 6, you will compare the growth patterns on the two different types of media. Before you begin, put on your safety gear (lab coat, safety glasses, and gloves).

1. **Situate Your Workspace:** Obtain your required materials and situate your workspace so it is not cluttered.
2. **Describe All Observed Growth:** Working with your group, examine the slants and describe the different growth patterns on the data sheet at the end of this lab exercise. Include a sketch of a representative portion of each sample. Use the uninoculated tube for comparison if growth is difficult to see on any of the slants.
3. **(Optional) Compare Growth to Prior Plates:** Compare the slant growth to the colonies on the corresponding agar plates from Exercise 6.
4. **Clean up:** Unless you are told to save the samples, discard the slants as directed by your instructor.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 7

DATA SHEET

Growth Patterns on Slants

During the Lab

1 Describe the growth on your slants, including margin, texture, and color. Draw a picture of a representative segment of each observed slant culture and include relevant information about the incubation conditions (e.g., time, temperature, and the medium used).

Organism	Description and Sketch
Control Tube	

2 Based on your observations, discuss similarities between the slant cultures observed in this exercise and the plated cultures you observed in Exercise 6. If you did not perform Exercise 6, look back at the materials and procedure for that lab exercise and hypothesize what similarities you may have observed.

Lab Related Questions

1 Which of the following is (are) false regarding slant cultures? (Select all that apply.)

a. They can be used to maintain a pure culture.
b. They can be used to observe bacterial pigmentation.
c. They can be used to isolate a pure culture.
d. They cannot be used to observe bacterial texture.
e. They are commonly used in biochemical tests.

2 Match the following:

_____ Filiform	a. Produces colored growth
_____ Spreading edge	b. Smooth texture with solid edge
_____ Transparent	c. Solid growth seeming to radiate outward
_____ Friable	d. Easily transmits light
_____ Pigmented	e. Rough texture with crusty appearance

3 The surface of a slant culture is usually inoculated using a:

a. streak isolation pattern.
b. stab inoculation with an inoculating needle.
c. fishtail inoculation with an inoculating loop.
d. drop of broth culture from a pipette.
e. sterilized glass rod.

Critical Thinking

1 Why is a bacterial slant impractical for isolating a pure culture?

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 7
DATA SHEET
(continued)

2 Why are agar slants an ideal way to maintain pure stock cultures?

— Lab Notes —

EXERCISE 8

Growth Patterns in Broth

After doing this exercise, you should be able to:

1. Describe morphological features of bacterial cultures cultivated in broth media.
2. Safely handle bacterial cultures and properly dispose of such cultures.

Background

Microorganisms cultivated in broth display a variety of growth characteristics. Some organisms float on top of the medium and produce a type of surface membrane called a **pellicle**. Others sink to the bottom as **sediment**. Some bacteria produce **uniform fine turbidity**, and others appear to clump in what is called **flocculent** growth. Others show pigmentation or form a ring at the surface (Figs. 8.1 and 8.2). Recall that broth media are typically inoculated with a pure culture and the bacteria are introduced to the media using an inoculating loop.

FIGURE **8.1** **Growth Patterns in Trypticase Soy Broth (TSB)** • **From left to right:** Uniform fine turbidity; Flocculent growth, notice the flakes within the turbidity; Pellicle, a film floating on surface. This pellicle's consistency is illustrated by the portions hanging down into the broth, an artifact of handling during the photo shoot, as is the sediment at the bottom, which fell from the pellicle at the surface; Uninoculated TSB for comparison; Ring at surface, with fine turbidity and a small sediment. Also note the purple coloration of the organism; Sediment.

Clinical Applications

Bacterial genera, and frequently individual species within a genus, demonstrate characteristic growth patterns in broth that provide useful information when attempting to identify an organism. Furthermore, many biochemical tests that are used to definitively identify bacteria are broth culture formats.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Sterile uninoculated brain–heart infusion broth (BHIB) control (1)
- ❑ Broth cultures of the following recommended bacteria:
  - ☐ *Bacillus subtilis*
  - ☐ *Corynebacterium xerosis*
  - ☐ *Kocuria rosea*
  - ☐ *Lactobacillus plantarum* or *Lactobacillus acidophilus*
  - ☐ *Micrococcus luteus*
  - ☐ *Mycobacterium smegmatis* (BSL-1 strains are available—i.e., ATCC 27203)
  - ☐ *Proteus mirabilis* (BSL-2)

FIGURE **8.2** **Pigmentation in Broth** • *Rhodospirillum rubrum* has a red color due to carotenoid pigments. It grows as a photoheterotroph in the presence of light and the absence of oxygen.

PROCEDURE

Today you will be examining the growth characteristics of up to seven different bacteria in broth. Before you begin, put on your safety gear (lab coat, safety glasses, and gloves).

1. **Situate Your Workspace:** Obtain your required materials and situate your workspace so it is not cluttered.
2. **Describe All Observed Growth:** Working with your group, examine the broth samples and describe the different growth patterns on the data sheet at the end of this lab exercise. Include a sketch of a representative portion of each sample. Use the uninoculated tube for comparison.
3. **Clean up:** Unless you are told to save the samples, discard the broth cultures as directed by your instructor.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 8

DATA SHEET

Growth Patterns in Broth

During the Lab

1 Describe the growth of your broth cultures. Draw a picture of a representative segment of each observed culture and include relevant information about the incubation conditions (e.g., time, temperature, and the medium used).

Organism	Description and Sketch
Control Tube	

Lab Related Questions

1 Which of the following is (are) false regarding broth cultures? (Select all that apply.)

a. They can be used to isolate a pure culture.
b. They can be used to observe bacterial pigmentation.
c. They will not allow for colony formation.
d. They cannot be used to observe bacterial texture.
e. They are commonly used in biochemical tests.

2 Match the following:

_____ Flocculent	a. Evenly cloudy throughout
_____ Sediment	b. Growth at top around the edge
_____ Ring	c. Suspended chunks or pieces
_____ Pellicle	d. Growth on the bottom
_____ Uniform fine turbidity	e. Membrane at the top

3 A broth culture is usually inoculated using:

a. A streak isolation pattern.
b. A stab inoculation with an inoculating needle.
c. A fishtail inoculation with an inoculating loop.
d. A sterilized glass rod.
e. An inoculating loop.

Critical Thinking

1 What factors besides physical growth characteristics of the organism are important when recording data about an organism? Why?

2 You have two 5 mL broth cultures that were inoculated at the same time with the same bacterium. Both were incubated for 24 hours at room temperature. One culture is slightly turbid and, at least to the naked eye, the second tube of media appears perfectly clear. You have only 48 hours to determine if any bacteria at all grew in the second tube. Describe two ways that you could determine if bacteria are present using only bacteriological culture tools. (***Note:*** Assume *both* experiments must be completed in their entirety within 48 hours, you do not get 48 hours per experiment.)

EXERCISE 9

Ubiquity of Microorganisms

After doing this exercise, you should be able to:

1. List and describe the types of symbiotic relationships microbes have with humans.
2. Define the terms *source* and *reservoir* and differentiate between the two.
3. Describe colony morphological features for bacteria isolated from an environmental sampling.
4. Sample environmental sources for microbiological life.
5. Safely handle and dispose of samples collected from an environmental source.

CAUTION!

The U.S. Centers for Disease Control and Prevention cautions, "Either nonpathogenic or attenuated bacterial strains should be used when possible, especially in teaching laboratories." Before assigning this exercise, faculty should check institutional policies regarding cultivating unknown bacteria from environmental sources and from human subjects. Anytime such sampling is performed, BSL-2 agents that are not attenuated could be isolated. Faculty are strongly cautioned to review their institution's policies on students handling such samples. This lab exercise should only be performed in laboratories approved to handle BSL-2 agents.

Background

Microorganisms have a long, rich history on Earth and have successfully adapted to a wide range of habitats. Microbes of all sorts can be isolated from soil (even desert sands), water (over a large range of salinities), plants, and animals (including humans). Microbes are even found in seemingly uninhabitable sites such as hot acid pools (Fig. 9.1). Microbes are often described as "ubiquitous in nature," meaning they are everywhere.

Many microorganisms are **free-living**, they do not require a specific **host** to survive. And although bacteria are often discussed in the context of disease, which makes it easy for us to assume the majority are **pathogens**, the truth is most are **nonpathogenic** (do not cause disease). Instead, most bacteria are **saprophytes** (live off dead/decaying matter) and therefore perform the important ecosystem role of breaking down organic matter. Many perform important conversions of inorganic molecules and participate in **biogeochemical cycles**, such as the sulfur or nitrogen cycles.

Other microorganisms reside on or in another species; they have a **symbiotic relationship** with their host(s). The microbes that normally live in and on us are a part of our **normal microbiota**, or **microbiome**. Microbes and humans have evolved a variety of symbiotic relationships, including those that hurt the host (**parasitism**), help the microbe and leave the host unaffected (**commensalism**), or are mutually beneficial to the host and the microbe (**mutualism**). That said, many of the commensal and mutualistic strains inhabiting our bodies may cause disease if introduced to body sites where they do not belong. For example, *Escherichia coli* is a natural inhabitant of the colon; we have a mutualistic relationship with these bacteria. We provide them with a food source and shelter, and they in turn help us digest certain foods and make vitamin K for us. However, when *E. coli* are displaced into an area where they do not belong, they can cause an infection (e.g., *E. coli* entering the urinary tract can cause a urinary tract infection). When an infectious agent that normally lives in or on your body is responsible for an infection, we say the infection was

FIGURE 9.1 ***Sulfolobus* in the Sulfur Caldron, Yellowstone National Park** • The pictured geologic feature of Yellowstone National Park makes abundant H_2S gas. *Sulfolobus* (domain Archaea) uses the H_2S as an energy source. These microbes oxidize the H_2S to sulfuric acid (H_2SO_4), which lowers the local environmental pH to 2 or less (about the equivalent of battery acid!). This extreme acidity breaks down rock and soil to produce the muddy conditions seen in the photo. The steam you see rising from the caldron is due to its high temperature. *Sulfolobus* species grow between 65°C and 85°C, that's roughly 150°F to 185°F! *Sulfolobus* usually gets its carbon from CO_2, just like plants.

derived from an **endogenous source**. Of course, other people, animals, and our environment are also potential pathogen sources, these are referred to as **exogenous sources**.

Environmental Sampling: A Cautionary Word

Environmental sampling is interesting and clinically useful, but it can be hazardous. Never perform environmental sampling unless your instructor expressly tells you to do so, and even then, only sample environments your instructor has approved.

You must apply strict caution whenever you're sampling the environment. After all, microbes in our environment infect us all the time, and that's *before* we purposely grow them, a process that concentrates their numbers and increases the infection risk (facilitates the pathogen reaching an **infectious dose** level). Because of these risks, many instructors only perform such exercises with microbiology majors in upper level courses. Some institutions have entirely forbidden such exercises for nonmajors and severely restrict the environments majors can sample (often body sites, bodily fluids, and facilities that contact such fluids are barred from sampling).

The bottom line is, if you sample the environment, there is a reasonable chance that you'll isolate a BSL-2 pathogen and concentrate it to an infectious dose level. Moreover, that isolated agent is likely to be fully **virulent** (able to exert its full disease-causing potential) as opposed to **attenuated** (weakened) like the strains we typically use in teaching lab exercises. As such, if you do perform such exercises, you must strictly follow *all* BSL-2 safety measures and only perform such exercises in a lab that is specifically designated as a BSL-2 facility (see the Introduction, pp. 2–4, for specifics on BSL-2 safety practices and facilities).

Clinical Applications

In this exercise you'll discover the ubiquitous nature of microorganisms. Even surfaces that appear clean are often covered in microbes. Our environment is an important source of infectious agents. A **source** is anything that spreads an infectious agent from its reservoir to a new host. A **reservoir** is any animate or inanimate habitat where a given pathogen is naturally found.

It's important to note that the terms *source* and *reservoir* are not synonyms. In some cases, the infectious agent's reservoir and the source of the infection are the same, but this is not a safe assumption. For example, humans are both the source and the sole reservoir for the smallpox virus. This fact made smallpox a terrific candidate for eradication. In contrast, *Clostridium botulinum*, the bacterium that cause botulism, naturally exists in soil. However, when people develop botulism, it is because they ingested a contaminated food (the source of the infection) versus directly ingesting soil (the natural reservoir). Discovering a pathogen's source and reservoir often requires environmental sampling.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Nutrient agar (NA) plates (8)
- ❑ Sterile cotton swab (1)
- ❑ Tube of sterile nutrient broth (NB) (1)

PROCEDURE

Today you'll sample your environment to observe what microbes are present and you'll qualitatively assess their relative abundance. Wear a lab coat, gloves, and eye protection when performing this procedure. If you are working in a lab group, then students in the group should share responsibility for inoculating the plates in this exercise.

1. **Situate Your Workspace:** Obtain your required materials and situate your workspace so it's not cluttered. Number the agar plates 1 through 8 (remember to label the base of the plates, not the lids).
2. **Plate 1:** Open Plate 1 and expose it to the air for 30 minutes or longer. Set it aside and out of the way of the other plates.
3. **Plates 2 and 3:** Dip your sterile cotton swab into the tube of sterile nutrient broth. Press the swab against the tube's inner wall to squeeze out excess media from the cotton tip. Then, use the damp swab to sample your desk/bench top; roll the swab on the

tabletop as you sample it. Once you have sampled the surface, streak Plates 2 and 3 in the pattern shown in Figure 9.2. As you streak the agar, gently roll the swab so you can ensure that the contaminated part of the swab contacts the agar. Also, use only light pressure so you don't cut into the agar. Dispose of the swab as your instructor directs.

4. **Plate 4:** With the lid off, hold Plate 4 directly in front of your mouth and cough several times onto the agar surface. Be sure you aren't facing anyone when you cough, instead, cough downward onto the plate to minimize spreading germs to others.
5. **Plate 5:** Rub your hands together, and then touch the agar surface of Plate 5 lightly with your fingertips. A light touch will do; touching too firmly will crack the agar. Your fingers will feel damp afterward, but the agar is sterile and safe to touch.

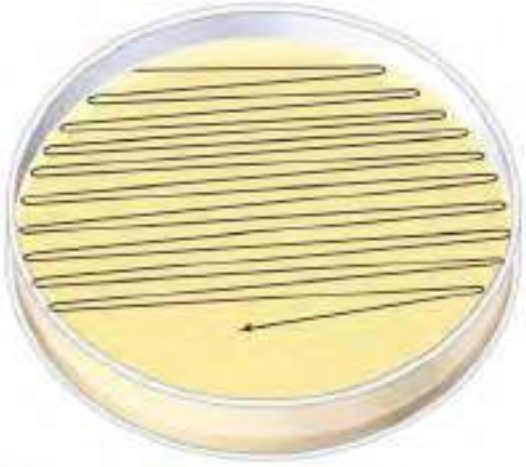


FIGURE **9.2** **Simple Streak Pattern on an Agar Plate** • Roll the swab as you inoculate the plate in a zigzag pattern that covers the entire plate surface. Do not press so hard that you cut the agar.

6. **Plate 6:** Remove the lid of Plate 6 and vigorously scratch your head above it. It is best to bend your head forward so your head/hair is above the plate. When inoculating, do so away from Plate 1 to avoid cross-contamination.
7. **Plates 7 and 8:** Leave Plates 7 and 8 covered; do not open them. These will be controls.
8. **Final steps:** Label the base of each plate with the date, the type of exposure it received, the incubation temperature (see Step 9), and your name.
9. **Incubate the plates:** Invert all plates and incubate them for 24 to 48 hours as follows:

   Plates 1, 2, and 8: incubate at 25°C

   Plates 3, 4, 5, 6, and 7: incubate at 35 ± 2°C

→ **Next Lab Session**

1. Remove your plates from the incubators and examine them for growth.
2. Record your observations and answer the data sheet questions at the end of this lab.

CAUTION!

Do not remove any of your plates' lids; you have cultivated unknown microbes and they should remain contained. Record your observations on the data sheet at the end of this lab exercise.

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 9

DATA SHEET

Ubiquity of Microorganisms

During the Lab

1 Describe the growth of your samples. For each plate, choose two different colonies and draw each as seen from above and from the side. Simple line drawings are acceptable. Record the incubation time, temperature, and source of inoculum for each plate. Also include other useful colony information, such as color and relative abundance.

Organism	Colony Descriptions and Sketches
Control plates (7 and 8)	
Plate 1	
Plate 2	
Plate 3	
Plate 4	
Plate 5	
Plate 6	

Lab Related Questions

1 The best definition of a reservoir is:

a. The place where a pathogen naturally exists.
b. The agent that spreads the pathogen from one host to another.
c. The place where a host becomes infected with a given pathogen.
d. The infectious agent that causes a particular disease.
e. The place a given pathogen reproduces.

2 Assume you discover a prokaryotic pathogen that naturally resides on human skin. This agent is discovered to cause urinary tract infections, but only in immune compromised people. What term(s) appropriately describe(s) this newly identified agent and/or the infection it causes? (Select all that apply.)

a. This agent could be described as a fungal pathogen.
b. This agent would be described as an opportunistic pathogen.
c. This agent would be described as a true pathogen.
d. The described infection can be derived from an endogenous source.
e. The described infection can be derived from an exogenous source.

3 Match the following:

_____ Exogenous source	a. Microbes that naturally reside in and on us.
_____ Endogenous source	b. Microbes that cause disease in a compromised host.
_____ Pathogen	c. An infectious agent contracted from the host's body
_____ Opportunistic pathogen	d. A microbe that causes disease in an otherwise healthy host.
_____ Microbiome	e. A microbe contracted from outside the host's body.

4 A pathogen would be best described as having a _____ relationship with its host.

a. mutualistic
b. commensal
c. parasitic
d. exogenous source
e. opportunistic

5 Assume you isolate a bacterium from a lake water sample. This bacterium is found to live outside a host, but it can be swallowed by ducks as they drink lake water. The bacterium readily thrives in the duck gastrointestinal tract and seems to benefit from the association. Ducks who carry the bacterium are usually protected from a rare fungal disease that affects water fowl. Based strictly on this information, select the applicable statements from below.

a. This bacterium exhibits a mutualistic relationship with ducks.
b. This bacterium exhibits a commensal relationship with ducks.
c. This bacterium is free-living.
d. This bacterium is important in biogeochemical cycles.
e. This bacterium is a pathogen.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 9
DATA SHEET
(continued)

6 Consider the fungus *Candida albicans*. This fungus naturally resides in and on the human body and is known to cause disease (candidiasis) when the host's normal microbiota is disrupted. Referring to the background section of this lab, provide three terms and/or phrases that appropriately describe this agent.

Critical Thinking

1 How would growth on the uninoculated plates affect your interpretation of the other plates?

2 The plates you are using for this lab will be autoclaved to completely sterilize them. The measures taken to disinfect the tabletops (the source of the organisms on Plates 2 and 3) are not as extreme. Why?

— Lab Notes —

EXERCISE 10

Standard Plate Count

After doing this exercise, you should be able to:

1. Perform a standard plate count to determine the cell density of a stock bacterial sample.
2. Discuss the uses and limitations of the standard plate count method.
3. Define the terms *countable*, *TNTC*, and *TFTC* as they apply to the standard plate count method.
4. Aseptically perform serial dilutions.
5. Calculate dilutions and original cell density.
6. Convert from µL to mL and vice versa.

Background

Microbiology is the science of extraordinarily small organisms in extraordinarily large numbers, which makes counting them a challenge on two fronts! Fortunately, we don't need to count all the cells in a sample to learn how many of them we most likely have. Instead, we only need to count the cells in a known fraction of the sample and then use some basic calculations to determine how many cells are most likely in the original sample.

The **standard plate count method** (also called the viable plate count method) is a common and inexpensive procedure for determining how many living cells are in a liquid sample. The fact that we are only counting living cells is important because it sets this **direct count** enumeration procedure apart from counting methods that detect the number of cells in a sample, but does not differentiate living from nonliving cells. While this is certainly an advantage of the standard plate count method, there are some drawbacks to the method.

The major disadvantage is in dealing with mixed cultures. There are not universally optimal conditions that equally promote the growth of all species in a mixed sample, so growth conditions are bound to favor one species over another. Consequently, this method won't accurately enumerate all the microorganisms in a mixed culture; some species may not even grow under the prescribed growth conditions. Instead, we select the species we want to enumerate (or group of species with the same growth requirements) and optimize the procedure to enumerate those microbes. Notably, this disadvantage becomes an advantage when we want to enumerate a specific bacterial population, such as bacterial groups associated with fecal contamination of food or water (i.e., enterococci).

Recall in the streak plate isolation method (Ex. 4) we discussed the concept of **colony-forming units**, or **CFUs**. Here we again rely on the concept that a single cell, when sufficiently separated from neighboring cells on an agar plate, will generate one colony. Of course, we can't guarantee that every colony came from exactly one cell, some colonies could develop from two cells stuck together or groups of cells adhering to one another based on the organism's typical cellular arrangement. Therefore, rather than saying a single cell will produce a colony, it's more precise to say that one CFU will produce one colony. That said, microbiologists often use CFU/mL and cells/mL interchangeably but, they do so with a full understanding of the reviewed limitations.

In the standard plate count method, we plate diluted portions of a sample onto agar plates and then count the number of colonies that grow. The number of resulting colonies is then used to calculate a sample's **original cell density** (OCD), which is expressed as CFU/mL.

Performing and Calculating Serial Dilutions

As shown in Figure 10.1, a serial dilution is a sequence of controlled transfers down a line of dilution blanks, tubes containing a known volume of sterile diluent (e.g., water, saline, or buffer). The dilution series begins with a sample of unknown cell concentration (density) and ends with a very dilute mixture containing only a few—or no—cells. Each dilution blank in the series receives a known volume from the previous tube and delivers a known volume to the next, typically reducing (diluting) the cell density by 10- or 100-fold at each step.

As an example, let's assume the original broth culture in Figure 10.1 contains 1,000,000 CFU/mL. Following the first transfer, which is a 100-fold dilution (10 µL of sample added to 990 µL of diluent), Tube 1 would contain 10,000 CFU/mL (calculation: 1,000,000/100 = 10,000). Upon performing another 100-fold dilution into Tube 2, we would have 100 CFU/mL (calculation: 10,000/100 = 100). Therefore, if we were to inoculate 1 mL of the sample in Tube 2, we'd expect about 100 colonies to grow. Because the original sample's cell density is unknown, only the dilutions are recorded on the tubes. By convention, dilutions are expressed in scientific notation. Therefore,

a 10-fold dilution (or a 1/10 dilution) is written as 10^{-1} and a 100-fold dilution (also noted as 1/100 dilution) is written as 10^{-2}.

In Figure 10.1, you can see that the first transfer in the series is a simple dilution while successive transfers are compound dilutions. Both types of dilutions can be calculated using the following formula:

$$V_1D_1 = V_2D_2$$

In this formula, V_1 and D_1 are the starting volume and dilution, respectively. In contrast, V_2 and D_2 are the new (or resulting) volume and dilution.

Let's work through how to calculate the dilution in Tube 1 of Figure 10.1. In such a case, D_2 is the dilution we want to calculate (the new dilution), so that is the variable we need to isolate. Here's what the formula looks like when we isolate D_2 on one side of the equation:

$$D_2 = \frac{V_1D_1}{V_2}$$

Next, we plug in our known values. In this case we know V_1, D_1, and V_2:

- V_1 is 10.0 µL (the volume of the original non-diluted sample that we transferred into Tube 1).
- D_1 is the starting dilution (we are starting with a non-diluted sample, so $D_1 = 1.0$).
- V_2 is our final volume (10 µL of sample were to 990 µL of diluent, so Tube 1 contains a final volume of 1,000 µL).

$$\frac{10.0\ \mu L \times 1.0}{1{,}000\ \mu L} = \frac{10}{1{,}000} = 0.01 = 10^{-2}$$

That's nice, but what does 10^{-2} (or 0.01) mean? How much did we ultimately dilute the original sample? Well, if we started with a non-diluted sample at full concentration (1.0) and we now have a dilution of 0.01, then we made a 100-fold dilution. How do we know that?

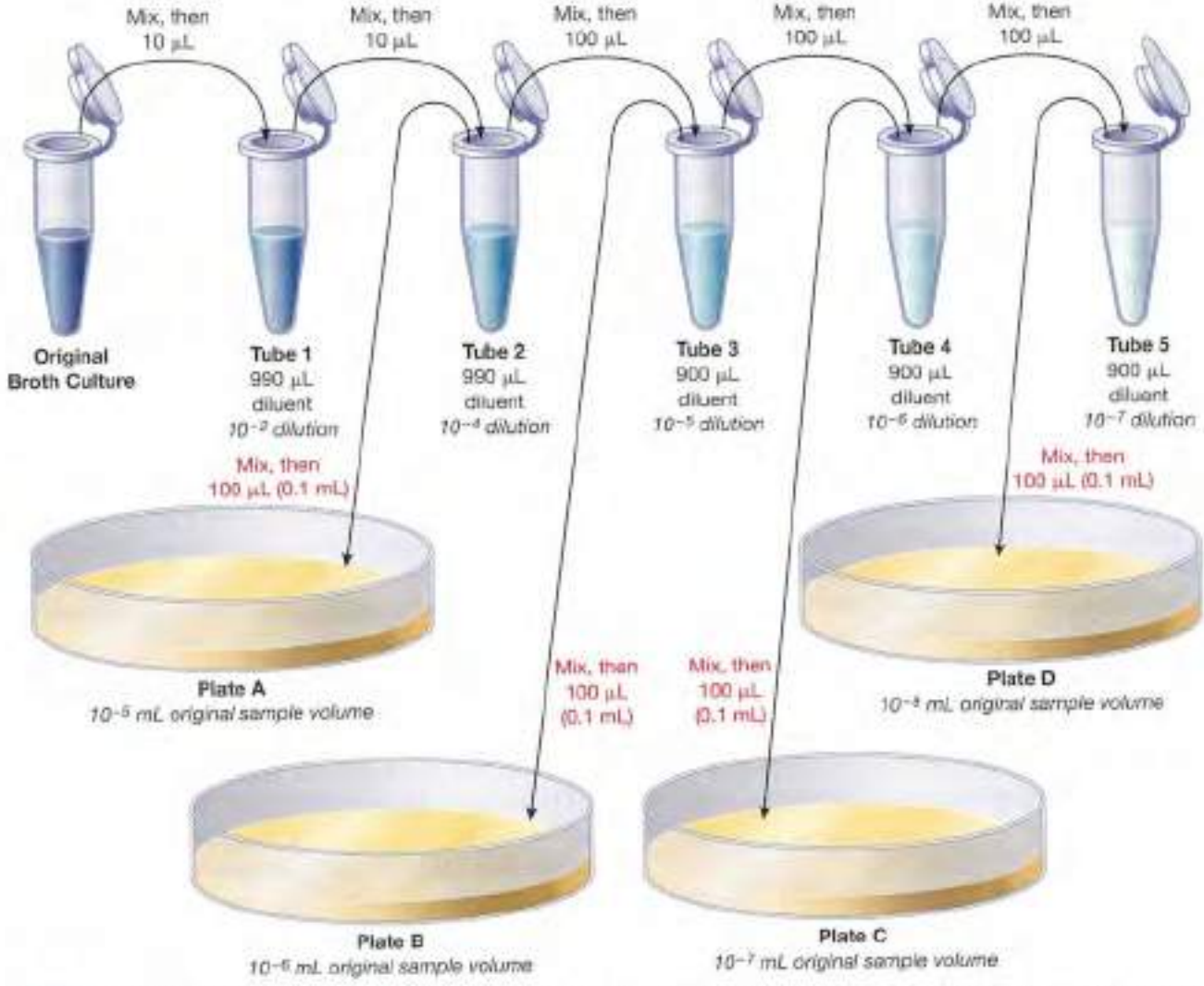

Mix, then 10 µL
Mix, then 10 µL
Mix, then 100 µL
Mix, then 100 µL
Mix, then 100 µL
Original Broth Culture
Tube 1 990 µL diluent 10⁻² dilution
Tube 2 990 µL diluent 10⁻⁴ dilution
Tube 3 900 µL diluent 10⁻⁵ dilution
Tube 4 900 µL diluent 10⁻⁶ dilution
Tube 5 900 µL diluent 10⁻⁷ dilution
Mix, then 100 µL (0.1 mL)
Mix, then 100 µL (0.1 mL)
Mix, then 100 µL (0.1 mL)
Mix, then 100 µL (0.1 mL)
Plate A
10⁻⁵ mL original sample volume
Plate D
10⁻⁸ mL original sample volume
Plate B
10⁻⁶ mL original sample volume
Plate C
10⁻⁷ mL original sample volume

FIGURE **10.1** **Serial Dilution Procedural Diagram** • This diagram shows the dilution scheme you will follow for the lab procedure. The dilution assigned to each tube (written below the tube) represents the proportion of original sample inside that tube. For example, if the dilution is 10^{-1}, the proportion of original sample inside the tube is 1/100th of the total volume.

$$\text{Fold dilution} = \frac{\text{Starting dilution}}{\text{Final dilution}} = \frac{1.0}{0.01} = 100$$

We can also calculate compound dilutions, such as the dilutions for the samples in Tubes 2 through 5 in Figure 10.1. In the first example we worked through, D_1 had a value of 1.0 to reflect it was not diluted (it was the original sample at full concentration). However, in this new scenario we are starting with a diluted sample, so D_1 is less than 1.0. Earlier we determined that Tube 1 contained a 0.01 or 10^{-2} dilution, this will therefore be our starting dilution, D_1. As an example, let's calculate the dilution in Tube 2 using the volumes reflected in Figure 10.1.

$$D_2 = \frac{V_1 D_1}{V_2} = \frac{10.0\ \mu L \times 0.01}{1{,}000\ \mu L} = 0.0001 = 10^{-4}$$

Counting Plates and Calculating Cell Density

Once all dilutions are prepared, the next step is to inoculate agar plates with a portion of the diluted samples. Not every dilution will be inoculated; the dilutions selected for inoculation and the volume inoculated depends on the original sample's *estimated* cell density. Ultimately, the goal is to generate at least one **countable plate**, a 100 mm agar plate that contains between 30 and 300 colonies (Fig. 10.2). A plate with fewer than 30 colonies is considered statistically unreliable and described as **TFTC** (Too Few to Count). A plate with more than 300 colonies is too dense to reliably obtain separated colonies and is categorized as TNTC (Too Numerous to Count).

There are several methods available for counting colonies on agar plates. A cheap yet effective approach is to use a handheld tally counter and a felt-tip marker. As colonies are counted, you simply mark each colony on the plastic base and simultaneously click the counter. This is the method most teaching labs will use, but more sophisticated counting methods do exist. For example, there are electronic pens that, when touched to the plastic petri dish below a colony, will record a tally. There are also software systems that capture an image of the plate and then allow a computer to count the colonies. Once all the plates containing between 30–300 colonies are counted, you can perform some simple calculations to determine the sample's original cell density (OCD), which is expressed as CFU/mL. The basic formula for this calculation is:

$$\text{OCD} = \frac{\text{CFU}}{D \times V}$$

In the bottom line of this equation (the denominator), we multiply $D \times V$. This is mathematically equal to the volume of the *original* sample represented on the plate that was counted. So, another way to write this equation is as follows:

$$\text{OCD} = \frac{\text{CFU}}{\text{Original sample volume (mL)}}$$

Next, we plug in our known values. For this example, assume we are using inoculum from Tube 3 in Figure 10.1:

- CFU is the number of colonies on the plate. For this example, let's assume it is 37.
- If we don't know the original sample volume that was inoculated onto the plate, we can calculate it by multiplying $D \times V$.
  - Since Tube 3 is a 10^{-5} dilution, we will plug in 0.00001 for D.
  - V is volume (expressed in mL) of the inoculum added to the agar plate. In Figure 10.1, we used 0.1 mL of sample from Tube 3 to inoculate the agar plate. ***Calculation note:*** In previous calculations where we were determining the dilution, we did not need to convert the volume from µL to mL. However, because OCD is expressed as cells per mL (cells/mL), we must convert all volume measurements to mL. This is done by moving the decimal to the left three place values. Mathematically, this is the same as dividing the µL amount by 1,000. When we do this, the 100 µL inoculated from Tube 3 becomes 0.1 mL.

$$\text{OCD} = \frac{37}{0.00001 \times 0.1\ \text{mL}} = \frac{37}{0.000001\ \text{mL}} = 37{,}000{,}000$$

$$= 3.7 \times 10^7\ \frac{\text{CFU}}{\text{mL}}$$

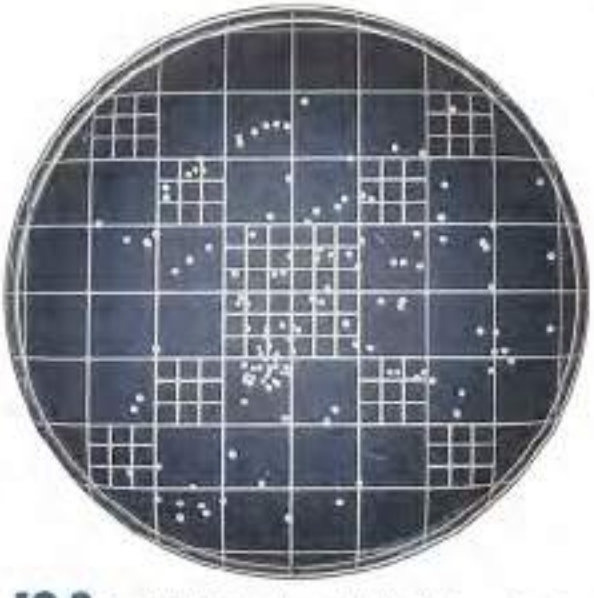


FIGURE **10.2 Countable Plate** • A countable plate, like the one shown here on a colony counting grid, has between 30 and 300 colonies. Plates with fewer than 30 colonies are TFTC ("too few to count"). Plates with more than 300 colonies are TNTC ("too numerous to count").

Clinical Applications

The standard plate count method is one of the most common methods used to obtain an estimate of living CFU density in samples. This method is especially important in keeping our food, drug, cosmetics, and water supplies safe. For example, when you see a "boil water advisory," it means that the water management company has detected an unsafe level of bacteria in our water and we must boil the water to render it safe to drink. While keeping our water safe to drink may seem like a small matter, it is a huge global concern. The World Health Organization estimates that each year about 3.575 million people die from water-related infections, making this the leading cause of death in the world.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Micropipettes (10–100 µL and 100–1,000 µL) with sterile tips
- ❑ Sterile microtubes (5)
- ❑ 50 mL flask with about 10 mL of sterile water or sterile saline
- ❑ Nutrient agar (NA) plates (8)
- ❑ Jar (and its lid) containing ethanol, cotton in the bottom, and a bent glass rod
- ❑ Hand-tally counter
- ❑ Colony counter (optional)
- ❑ 24-hour broth culture of *Escherichia coli* (contains 2×10^7 to 2×10^{10} CFU/mL)

PROCEDURE

In this exercise, you will perform a dilution series and determine the population density of an *Escherichia coli* broth culture. To accomplish this, you'll inoculate the plates using the spread plate technique, as described in Exercise 5. As illustrated in Figure 5.4, page 82, the inoculum will be evenly dispersed over the agar surface with a bent glass rod. *Safety Note:* You will be sterilizing the glass rod between inoculations by immersing it in alcohol and then igniting it to dry off the alcohol. Organize your work area so the flame is kept away from the alcohol jar. If a fire starts in the jar, replace the jar's lid to extinguish the flame.

Refer to the procedural diagram in Figure 10.1 as needed. Appendix E provides an alternative procedure using glass pipettes and mL volumes.

1. **Situate Your Workspace:** Obtain all required materials (to include an *E. coli* sample and a flask of sterile saline or sterile water) and situate your workspace so it's not cluttered.
2. **Prepare Five Dilution Tubes:** Label your five microtubes 1–5. Once you have done this, aseptically add 990 µL sterile water/saline to Dilution Tubes 1 and 2. Close the caps when finished. Aseptically add 900 µL sterile water/saline to Dilution Tubes 3, 4, and 5. Close the caps when finished.
3. **Add Bacteria to Dilution Tube 1:** Carefully, but thoroughly, mix the stock bacterial culture and aseptically transfer 10 µL of the stock to Dilution Tube 1; mix well. This is a 10^{-2} dilution.
4. **Add Bacteria to Dilution Tube 2:** Aseptically transfer 10 µL from Dilution Tube 1 to Dilution Tube 2; mix well. This is a 10^{-4} dilution.
5. **Add Bacteria to Dilution Tube 3:** Aseptically transfer **100 µL** (*Note:* This is *not* **10 µl** as you used in Step 4!) from Dilution Tube 2 to Dilution Tube 3; mix well. This is a 10^{-5} dilution.
6. **Add Bacteria to Dilution Tube 4:** Aseptically transfer 100 µL from Dilution Tube 3 to Dilution Tube 4; mix well. This is a 10^{-6} dilution.
7. **Add Bacteria to Dilution Tube 5:** Aseptically transfer 100 µL from Dilution Tube 4 to Dilution Tube 5; mix well. This is a 10^{-7} dilution.
8. **Label Eight Plates:** Obtain eight plates, organize them into four pairs, and label them A_1, A_2, B_1, B_2, etc.
9. **Inoculate Your Plates:** After mixing, aseptically transfer 100 µL from Dilution Tube 2 to Plate A_1. Using the spread plate technique (Ex. 5), disperse the inoculum evenly over the entire surface of the agar. Repeat the procedure with Plate A_2, and label both plates "10^{-5} mL original sample," or simply "10^{-5}." Following the same procedure, transfer 100 µL volumes from Dilution Tubes 3, 4, and 5 to Plates B_1 and B_2, C_1 and C_2, and D_1 and D_2, respectively. Label the plates with their appropriate original sample volumes.
10. **Incubate the Plates:** Let the liquid inoculum soak into each plate for a few minutes. Then, invert each plate and incubate them at 35 ± 2°C for 24 to 48 hours.

11 **Dispose of Waste:** Save or dispose of the original *E. coli* stock culture as your instructor directs. Tightly cap the dilution tubes and dispose of them in a biological waste container to be autoclaved.

→ Next Lab Session

1 **Count the Colonies:** After incubation, examine the plates and determine if any are "countable" (plates with 30 to 300 colonies). Plates with less than 30 colonies are considered "too few to count" (TFTC) while those with more than 300 colonies are regarded as "too numerous to count" (TNTC). Count only the colonies on your countable plates. If available, a colony counter (Fig. 10.3) can be helpful for this.

2 **Calculate Original Cell Density (OCD):** After counting the colonies on each countable plate, average your numbers. You'll use the colony count average to determine your stock sample's OCD. The formula for this calculation was reviewed in the lab's background section and it's presented again in the data sheet.

3 **Dispose of Your Plates:** When finished, dispose of the plates in an appropriate autoclave container.

FIGURE **10.3** **Counting Bacterial Colonies** • Place the plate upside down on the colony counter. Turn on the light and adjust the magnifying glass until all the colonies are visible. Using the grid in the background as a guide, count colonies one section at a time. Mark each colony with a felt-tip marker as you record with a hand-tally counter.

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 10
DATA SHEET

Standard Plate Count

During the Lab

1 Count the number of colonies for each countable plate. For any plate containing more than 300 colonies, enter TNTC ("too numerous to count"). For plates containing fewer than 30 colonies, enter TFTC ("too few to count"). If no plates are countable, use the TFTC plate closest to 30 colonies for practice. If you must use a TFTC plate to practice the calculation, make a note of that in the table and in Question 2.

Plate	A_1	A_2	B_1	B_2	C_1	C_2	D_1	D_2
State if the plate was countable, TNTC, or TFTC								
For your countable plates, state how many colonies were counted*								

* If you did not get any countable plates, you may count your TFTC plates and use those numbers to practice all calculations.

2 Calculate the average number of colonies from the countable plates.

Average based on countable plates = _____ colonies

Did you need to use TFTC plates to practice this calculation? (Yes or No) _____

3 Calculate the original density in CFU/mL using the following formula:

$$\text{OCD} = \frac{\text{CFU}}{\text{Original sample volume}}$$

Original density of the *E. coli* stock culture (***Note:*** Express your answer as cells/mL)	

Lab Related Questions

1 The standard plate count is specifically used to _____.

a. enumerate the number of live cells in a sample
b. determine if a culture is pure or mixed
c. enumerate the total number of cells in a sample (living or dead)
d. identify selected pathogens in a sample
e. enumerate all live microbes in a sample

2 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ The standard plate count method is considered an indirect enumeration technique.

_____ The standard plate count method effectively enumerates all live bacteria in a sample.

_____ To perform the standard plate count method, a glass rod is used to spread a sample on an agar plate.

_____ A 100-fold dilution is the same as a 10^{-3} dilution.

_____ In the standard plate count method, a plate with 30 colonies would be considered countable.

3 In the standard plate count method, what do the following abbreviations mean?

OCD: _____

TNTC: _____

TFTC: _____

CFU: _____

4 A sample has a density of 1.37×10^5 CFU/mL. What sample volume(s) should yield a countable plate? (Select all that apply.)

a. 1.0 mL
b. 0.1 mL
c. 0.001 mL
d. 0.2 mL
e. 0.002 mL

5 Which of the following is a limitation of the standard plate count method? (Select all that apply.)

a. This method can only be used on pure cultures.
b. This method will not enumerate all bacteria in a sample.
c. This method cannot differentiate between living or dead cells in a sample.
d. This method can only count bacteria that we can grow in the laboratory setting.
e. This method is expensive and requires highly specialized equipment.

6 Assume you added 1 mL of a non-diluted sample into 999 mL of water. What is the final dilution?

a. This is a 1-fold dilution.
b. This is a 10-fold dilution.
c. This is a 100-fold dilution.
d. This is a 1,000-fold dilution.
e. There is not enough information provided to determine the final dilution.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 10 DATA SHEET

(continued)

Critical Thinking

1 You are testing two different wound dressing materials to determine if one reduces bacterial counts over the other. You obtain the standard plate count data in the provided table. Calculate the OCD for each material and decide if one material decreased bacterial growth as compared to the other. Show your work for each calculation and clearly indicate the calculated final OCD for each material. Assume that 0.001 mL of each sample was inoculated onto each plate.

Dilution	Colony Counts after 12 Hours of Incubation Material A	Colony Counts after 12 Hours of Incubation Material B
10^{-2}	Plate 1: 300	Plate 1: 301
	Plate 2: 214	Plate 2: 315
10^{-3}	Plate 1: 28	Plate 1: 30
	Plate 2: 26	Plate 2: 32

a. OCD of Material A (calculation):

b. OCD of Material B (calculation):

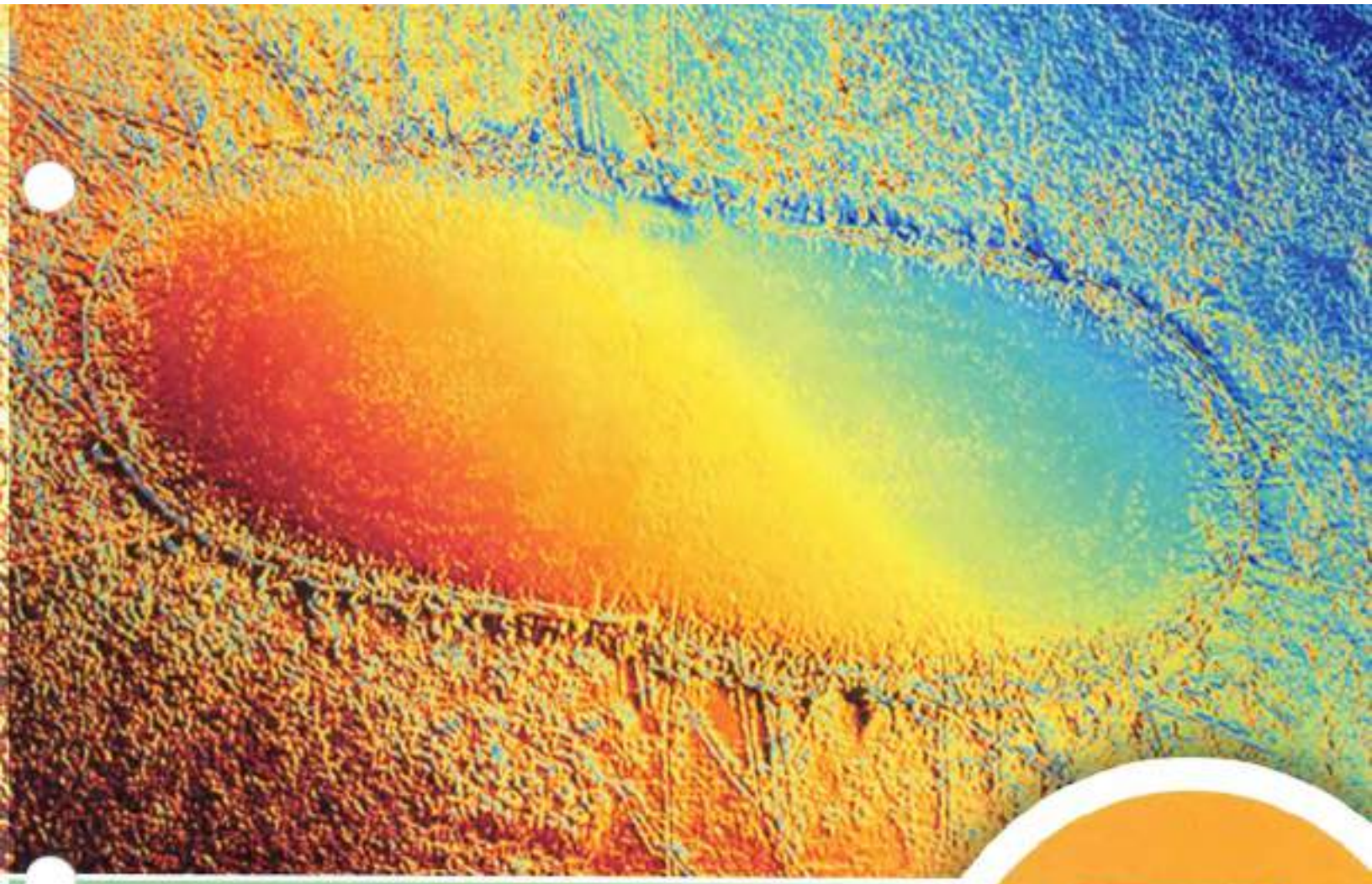
c. Did one material reduce bacterial counts over the other? If so, which one was "best"?

2 An ice-cream manufacturer, Sweet Treat, Inc., was sued because eight people died of a newly identified foodborne infection that was linked back to their Melon-Cream products. Sweet Treat, Inc., had a comprehensive microbiology quality control system in place and all evidence indicated that at the time the patients became ill, the manufacturer was in full compliance with the U.S. Food and Drug Administration's safety standard, which states:

7 CFR 58.648 — Microbiological requirements for ice cream.

"The finished product shall contain not more than 50,000 bacteria per gram as determined by the standard plate count, and shall contain not more than 10 coliform organisms per gram for plain and not more than 20 coliform per gram in chocolate, fruit, nut or other flavors in three out of five samples tested."

Sweet Treat's products were confirmed as the infection source, but the company was absolved of all liability for the infections because they were in full compliance with the FDA's regulations. Based on the case information and the information presented in the lab background about the standard plate count method, why does this ruling (even if it seems socially unfair) make sense?



SECTION

3

Staining Bacteria

In This Section

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INSTRUCTIONAL STAINING ATLAS

These figures represent some typical staining results for a variety of species. As you perform various staining labs in your microbiology course, you may wish to refer to these images for some guidance on interpreting stain results as well as deciphering cellular shapes and arrangements.

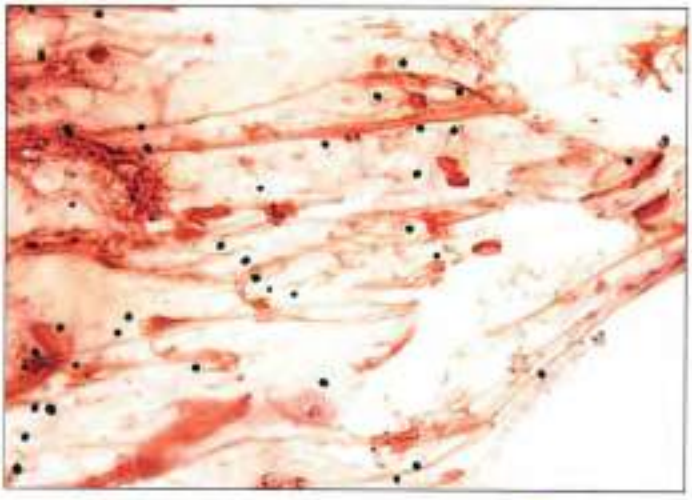


FIGURE IA.1 **Single Cocci from a Nasal Swab (Gram Stain)** • This direct smear of a nasal swab shows cocci (**dark circles**) stained with crystal violet. Each cell is about 1 µm in diameter. The red-orange background material is mostly mucus.

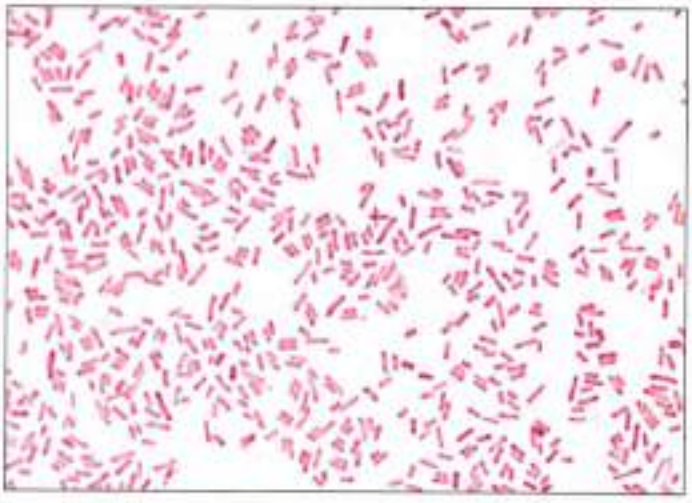


FIGURE IA.2 **Gram Stain of *Escherichia coli*, a Common Species in the Family *Enterobacteriaceae*** • All *Enterobacteriaceae* are Gram-negative, oxidase-negative rods. They also ferment glucose to acid end products.

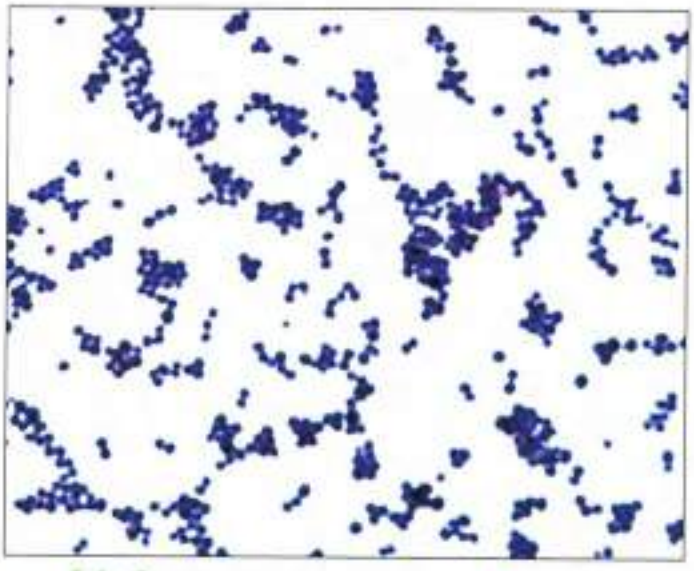


FIGURE IA.3 **Gram Stain of *Staphylococcus aureus*** • This specimen grown in broth illustrates the grapelike cell clusters characteristic of the genus. Specimens grown on solid media may not as clearly show cluster arrangements.

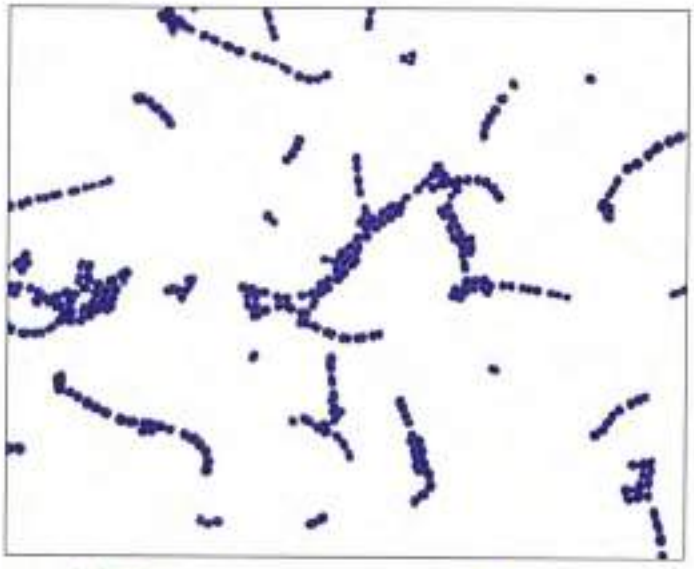


FIGURE IA.4 **Gram Stain of *Streptococcus salivarius*** • This specimen grown in broth illustrates the chainlike (streptococcal) cellular arrangement characteristic of the genus. Specimens grown on solid media may not as clearly show this cellular arrangement.

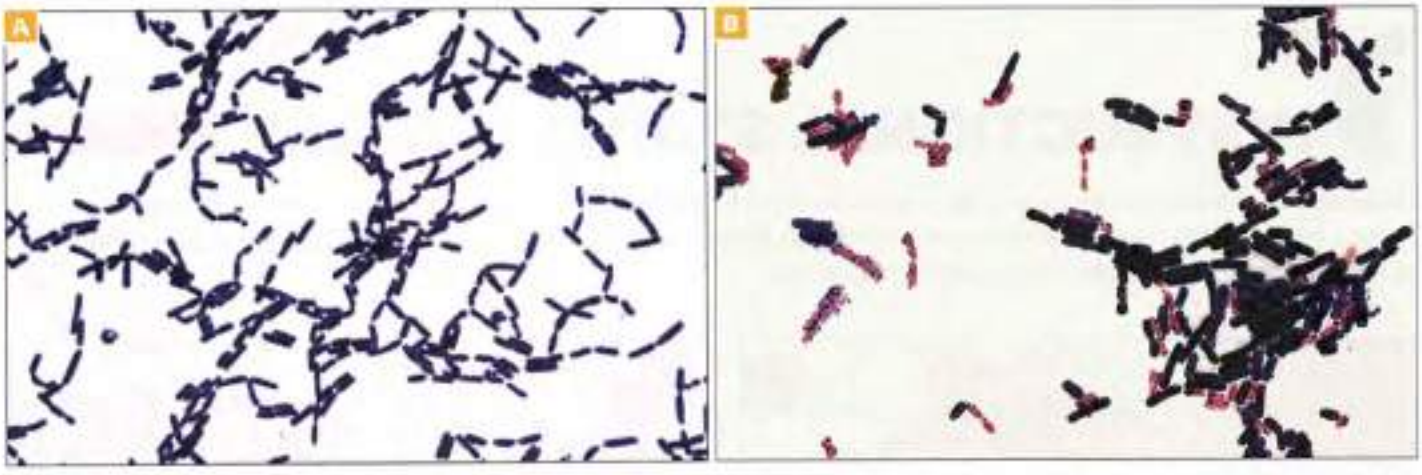


FIGURE IA.5 **Gram Stains of *Bacillus cereus*** • (A) This Gram stain of a young (<24 hours old) *B. cereus* culture stains uniformly Gram-positive. (B) With age, *Bacillus* species may undergo endospore formation and exhibit Gram variability, a situation in which some cells stain purple (Gram positive) and others appear pink (Gram negative). In this case, sporulating cells break down their cell wall and therefore do not retain crystal violet-iodine complexes during Gram staining.

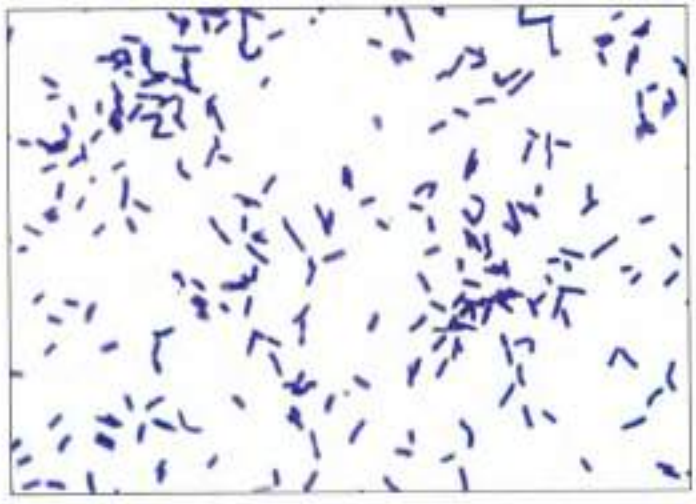


FIGURE IA.6 **Gram Stain of *Lactobacillus* spp.** • Cells in the genus *Lactobacillus* range from long, slender rods to coccobacilli.

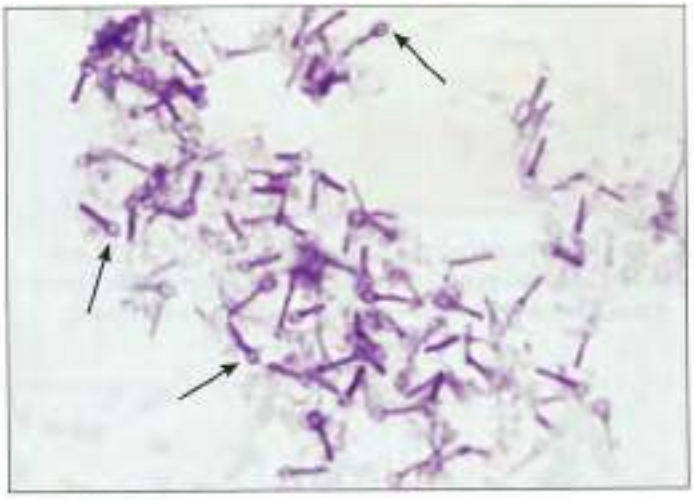


FIGURE IA.7 **Gram Stain of *Clostridium tetani*** • Note the round, terminal endospores (**arrows**) that distend the cells.

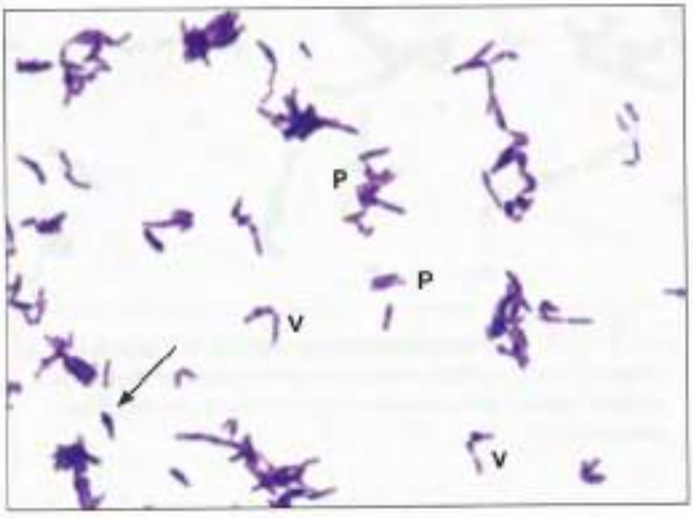


FIGURE IA.8 **Gram Stain of *Corynebacterium diphtheriae*** • Note the club-shaped cells that might be mistaken for spore producers (**arrow**). Cells of this genus often appear as "V-shaped" pairs (**V**) or palisades (**P**).

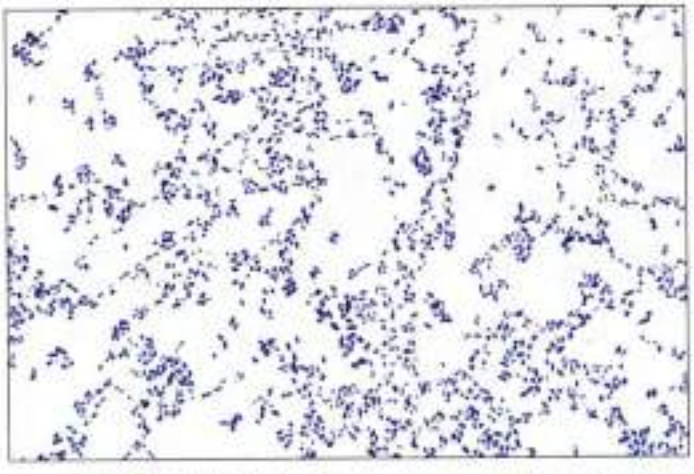


FIGURE IA.9 **Ovoid Cocci (Gram Stain)** • *Lactococcus lactis* is an elongated coccus that a beginning microbiologist might confuse with a rod. Notice the slight elongation of the cells, and that most cells are not more than twice as long as they are wide. *L. lactis* is found naturally in raw milk and milk products, but these cells were grown in culture. Cell dimensions are about 1–2 µm long by 1 µm wide.

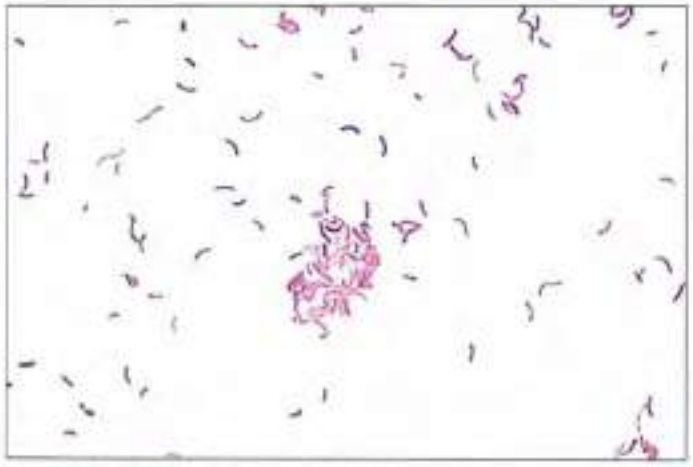


FIGURE IA.10 **Vibrio (Gram Stain)** • *Vibrio cholerae* is the causative agent of cholera. These cells often look like curved rods, hence the generic name, *Vibrio*. They range in size from 1–3 µm long by less than 1 µm in diameter.

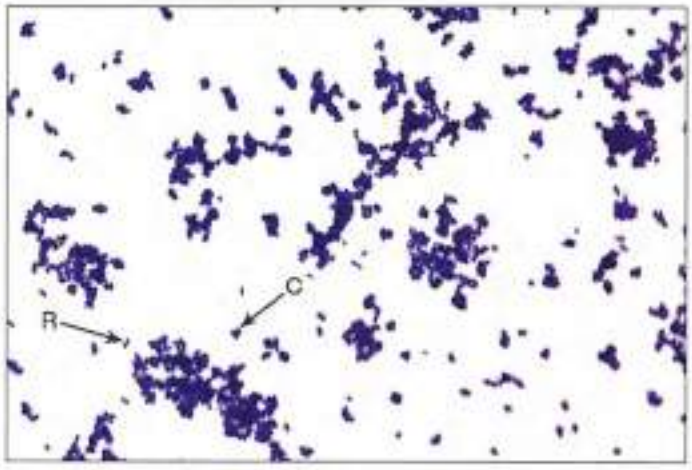


FIGURE IA.11 **Bacterial Pleomorphism (Gram Stain)** • Some bacterial species grow in a variety of shapes and are therefore described as pleomorphic (pleo = more than one; morphic = form). Here the rods of *Corynebacterium xerosis* range from plump cocci (**C**) to short, slender rods (**R**). *C. xerosis* is a normal inhabitant of skin and mucous membranes and is generally not considered a pathogen.

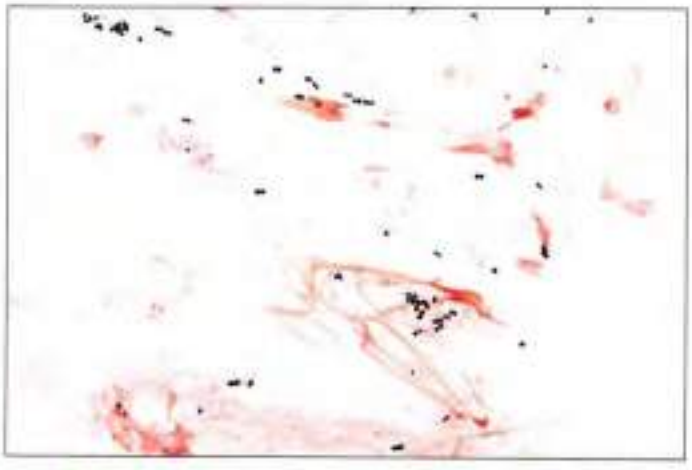


FIGURE IA.12 **Diplococcus Arrangement (Gram Stain)** • Most cells are in pairs, but a few are singles because they have yet to divide. Expect to see a mixture of cell arrangements representing stages before the "final" one.

FIGURE IA.13 **Tetrad Arrangement (Gram Stain)** • *Neisseria sicca* frequently grows in tetrads, as shown in this Gram stain of a trypticase soy agar culture. Cell arrangement (especially for cocci) is usually easier to determine from a specimen grown in a broth medium. This is because groups of cells separate from one another in liquid whereas they may artificially clump together when the bacteria are grown on a solid medium. That said, tetrads are usually easy to spot even when the bacteria are grown on a solid medium. Look at the bigger clumps and you will see tetrads within them. *N. sicca* is a normal inhabitant of the human nasopharynx, saliva, and sputum, and is an opportunistic pathogen. Cells are between 1 and 2 µm in diameter.

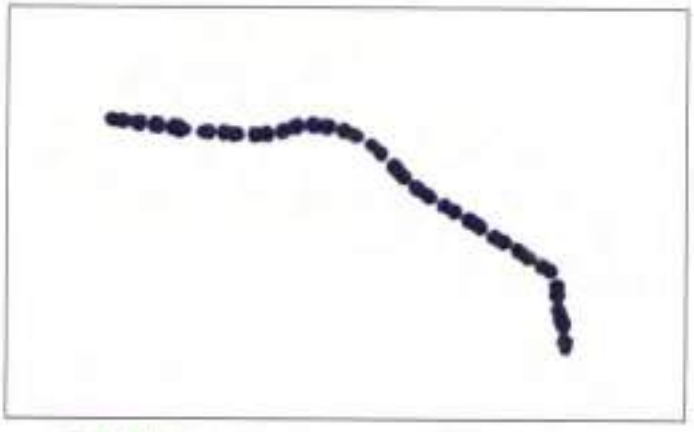


FIGURE IA.14 **Streptococcus Arrangement (Gram Stain)** • As its name suggests, *Streptococcus salivarius* forms long chains of spherical cells and inhabits the oral cavity (abundant in saliva and in the tongue). This specimen is from a broth culture. Individual cells are approximately 1 µm in diameter.

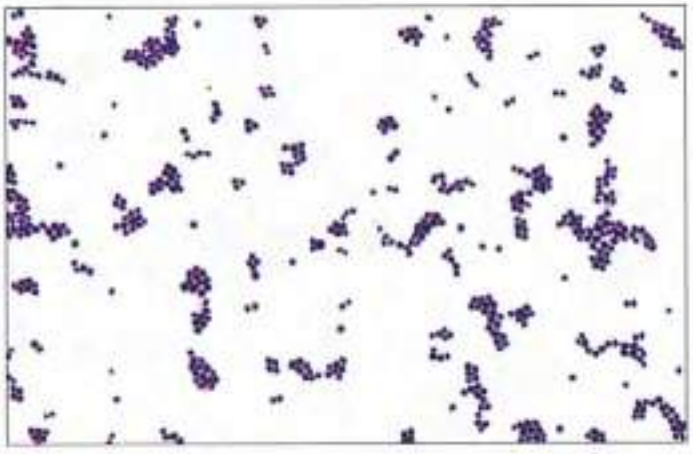


FIGURE IA.15 **Staphylococcus Arrangement (Gram Stain)** • *Staphylococcus aureus* cells grown in broth culture are shown. Note that the staphylococcal groups are rarely bigger than a dozen cells or so. Larger clusters are generally two or more adjacent groups. This "bunching" can be especially problematic when making a preparation of cells grown on a solid medium. Be sure to emulsify them thoroughly to spread clusters out as much as possible. *S. aureus* is a common opportunistic pathogen of humans. Cells are approximately 1 µm in diameter.

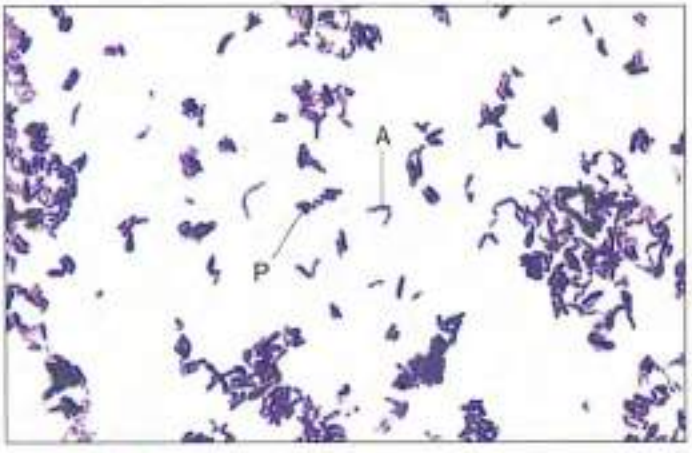


FIGURE IA.16 **Palisade and Angular Arrangements of *Arthrobacter* (Gram Stain)** • Stacking of rod-shaped cells side-by-side is the palisade arrangement (**P**). An angular arrangement (**A**) is when a pair of rods is bent where the cells join. Both arrangements are due to "snapping division" in which the cells divide lengthwise and remain more or less attached. *Corynebacterium* also shows these arrangements.

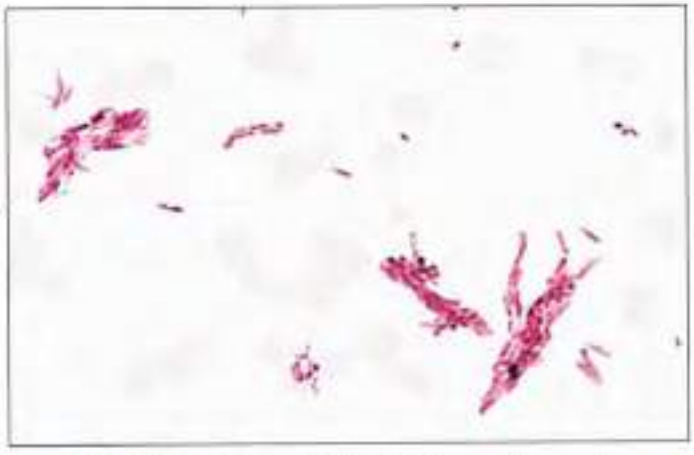


FIGURE IA.17 **Acid-Fast Stain of *Mycobacterium nonchromogenicum*** • *Mycobacterium* species are typically composed of slender rods with a "beaded" appearance (note the dark spots). Because of their waxy walls, they are difficult to emulsify and tend to clump.

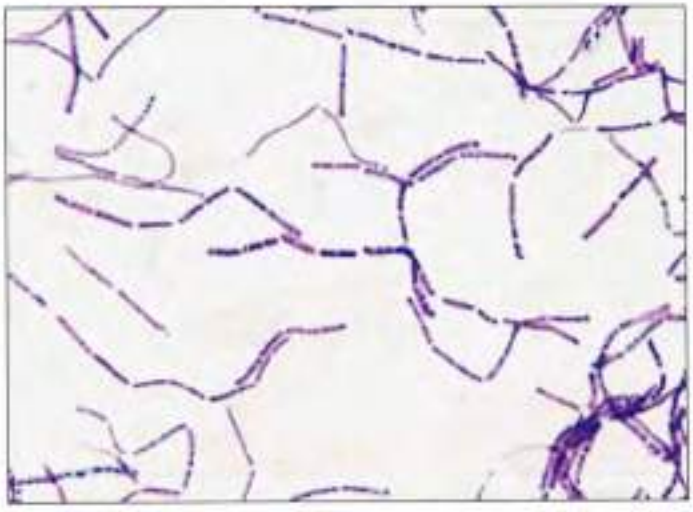


FIGURE IA.18 **Streptobacillus Arrangement (Crystal Violet Stain)** • *Bacillus megaterium* is a streptobacillus. These cells are 2–5 µm long by 1.2–1.5 µm wide.

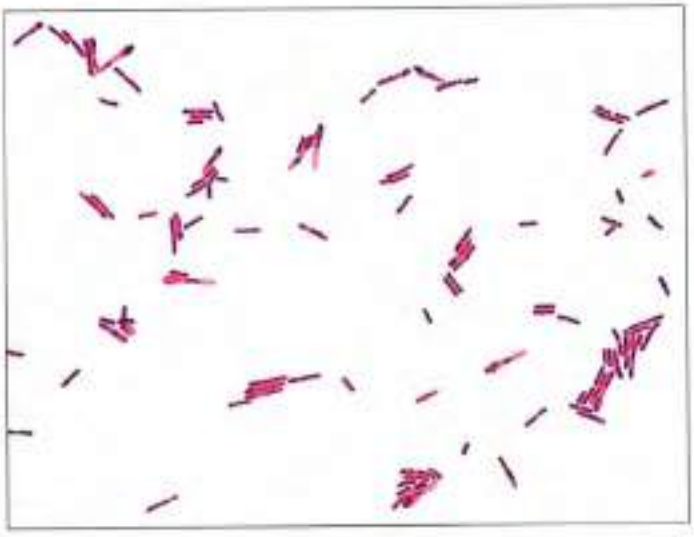


FIGURE IA.19 **Single Bacilli (Carbolfuchsin Stain)** • *Clostridium butyricum* tend to appear as single bacilli. It is the type species for the genus (meaning it was the first described *Clostridium* species back in 1880!). It is found in soil and aquatic environments, as well as in human feces and many other habitats. It produces endospores, which are visible in this image as bulges toward the ends of some cells. The cells range in size from 0.2–1.7 µm in diameter by 2.5–7 µm long.

FIGURE IA.20 Long, Thin Bacillus (Phase Contrast Wet Mount) • This *Bacillus subtilis* culture was grown in trypticase soy broth for 24 hours and is shown in a wet mount using high-dry (40× lens) for magnification. Cell morphologies can vary depending on the medium used (solid vs. liquid) and culture age. The longer "individuals" you see here are actually short chains of two or three cells, as evidenced by transverse constrictions or bends where the cells are attached. The water layer of this wet mount is considerably thicker than the cells, therefore cells above or below the focal plane appear out of focus.

FIGURE IA.21 Two Different Spirilla (Phase Contrast Wet Mount) • Based on their different cell morphologies, these spirilla (**arrows**) are undoubtedly different species. One is long and slender with loose spirals; the other is shorter and fatter with tighter coils. Cell dimensions are less than 1 µm wide and up to 35 µm long.

FIGURE IA.22 A Spirochete (Bright-Field Wet Mount) • Spirochetes (or spirochaetes) are tightly coiled, flexible rods. This bright-field micrograph shows a marine species of *Spirochaeta* (**arrow**). Notice the bend in the center of the cell. Cell dimensions of the genus are 5–500 µm long by less than 1 µm wide.

— Lab Notes —

EXERCISE 11

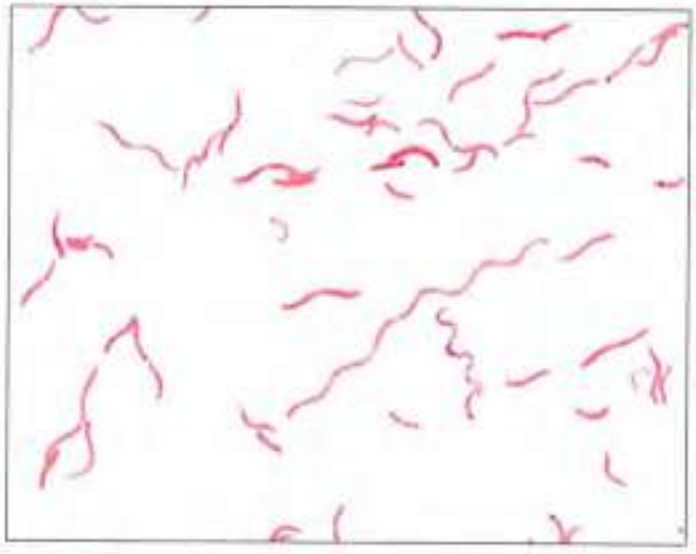
Simple Stains and Bacterial Cell Morphology

After doing this exercise, you should be able to:

1. Perform a simple stain of bacterial smears.
2. Describe and identify bacterial shapes and arrangements.
3. List the features of basic versus acidic dyes.
4. Discuss the role of fixation in staining procedures.
5. Perform oil-immersion microscopy to view your own stained bacterial samples.

Background

It can be difficult to see a clear microscopic specimen on a glass slide, so we use stains to add contrast to specimens. While microbes of all types can be stained, here we focus on how we can stain bacteria. There are a variety of bacterial staining procedures available. Some are classified as **structural stains**, which highlight specific structural features of a cell (e.g., the presence of endospores, a flagellum, or a capsule). Others serve as **differential stains** and allow us to separate one category of cell from another (e.g., differentiate between Gram-positive and Gram-negative cells). Then we have **simple stains**, which are the fastest and easiest way to add contrast to a specimen so it is easier to observe under the microscope. Simple stains can only reveal elementary morphological information, such as the bacterium's shape, arrangement, and size (Fig. 11.1).



11.1 Simple Stained Bacterium • This is a simple stain of the spiral-shaped bacterium *Rhodospirillum rubrum*. Notice that the stain is associated with the cells as opposed to staining the background. The organism was grown in a broth culture, stained using the basic stain safranin, and viewed with bright-field microscopy. This photograph was taken at 1,000× (oil immersion).

Bacterial Morphology: Shapes and Arrangements

Bacteria come in diverse shapes, arrangements, and sizes. Determining cell shape is an important first step in identifying a bacterial species. Cells may be spheres (**cocci**, singular **coccus**), rods (**bacilli**, singular **bacillus**), or spirals (**spirilla**, singular **spirillum**). Variations of these shapes include slightly curved rods (**vibrios**), short rods (**coccobacilli**), and flexible spirals (**spirochetes**). You can refer to Figure 11.2 and the Instructional Staining Atlas (pp. 131–135) for an overview of bacterial shapes. Although we do not fully understand why certain bacteria have a particular shape, we do know shape is a genetic trait.

In addition to describing bacterial shapes, we also note bacterial cell **arrangements** (the higher order arrangement of cells with respect to one another). Some species do not form higher order arrangements, in which case we describe them as **single**. Spirilla are almost always single cells, but cocci and bacilli often form multicellular associations.

If two daughter cells remain attached after a coccus divides, a **diplococcus** is formed. The same process happens in bacilli that produce **diplobacilli**. If the cells continue to divide in the same plane and remain attached, they form a chain and exhibit a **streptococcus** or **streptobacillus** arrangement. Cocci and bacilli can also divide to form clusters of cells. Cocci can divide to make **tetrads** (four cells clumped together), **sarcina** (groups of eight cells in a cubelike configuration), and **staph** arrangements (irregular grapelike clusters). In contrast, when bacillus-shaped bacteria form clusters we describe them as having a **palisade** arrangement. Figure 11.2 shows the main arrangements that bacterial cells assume.

When examining a specimen that forms higher-order cellular arrangements, it's common to see various stages of these arrangements. For example, if you are viewing a smear of staphylococci, you'll see single cells that broke off a larger cluster and have not yet divided into a staph arrangement; some cells that have divided into pairs; groups of four cells (tetrads); and staph arrangements, the final arrangement format. Be patient and look for the

most common morphology and the most complex arrangement to determine the organism's most likely true arrangement. If you have difficulty identifying cell shapes and/or arrangement, consider transferring the organism to a broth culture, incubating it (even for an hour or so), and trying again. Sometimes bacterial arrangements are easier to determine when the organisms are grown in a broth rather than on a solid medium.

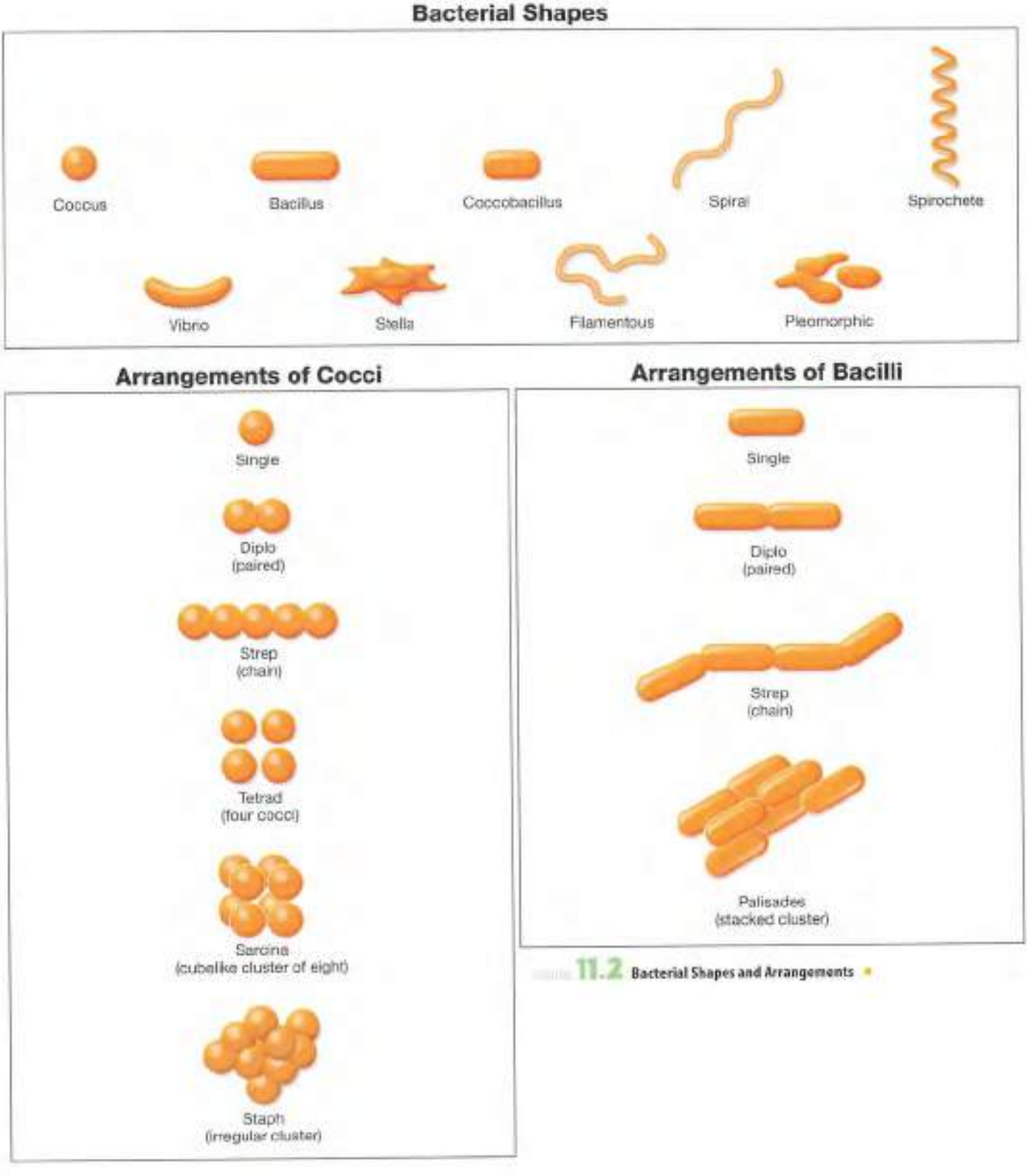


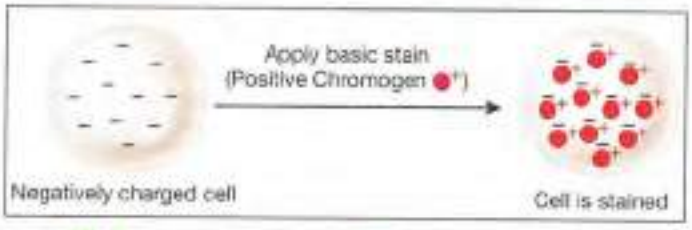
Figure 11.2 Bacterial Shapes and Arrangements

Basic Versus Acidic Dyes

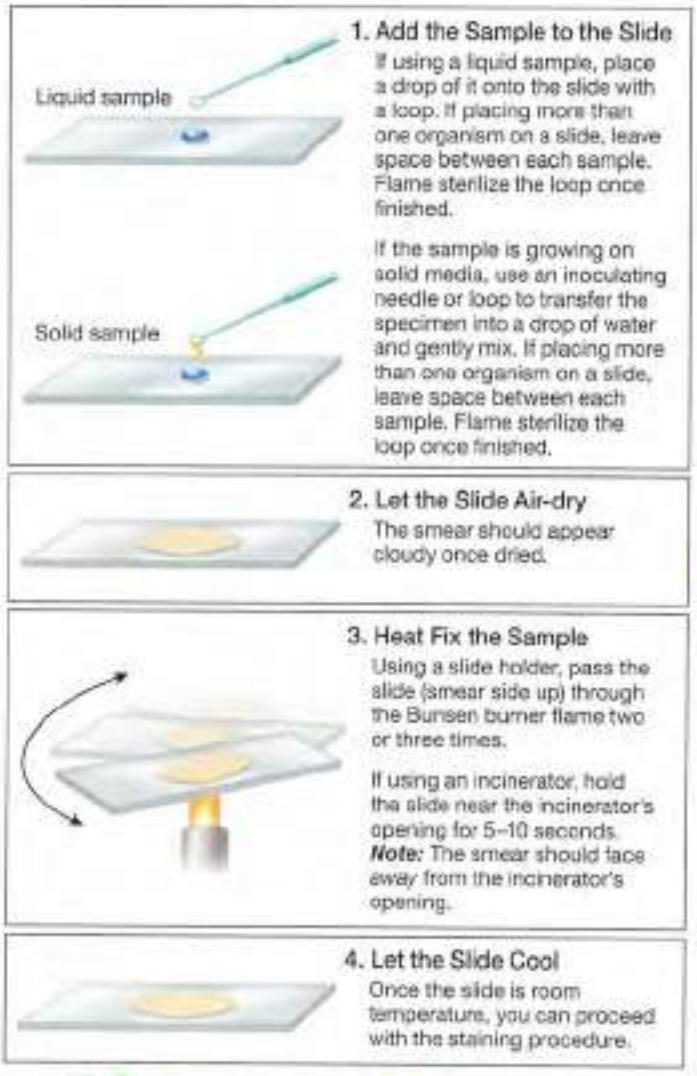
No matter what type of staining procedure is performed, a colored solution (stain) is added to the specimen. A staining solution contains a dye (or **chromagen**) dissolved in a solvent (usually water or ethanol). Simple stains are considered simple because they employ only one dye. The dye's chemical nature, especially its charge, affects how it interacts with cells. The adage "opposites attract" rings true in staining, namely a substance with a positive charge is attracted to a substance with a negative charge. Many common dyes such as methylene blue, crystal violet, and safranin are alkaline (are **basic**, or have a pH above 7) and have a positive charge. These positively charged dyes are readily attracted to and bound inside bacterial cells, which tend to have an overall negative charge (Fig. 11.3). In contrast, **acidic dyes** carry a negative charge and are repealed by bacterial cells, so rather than entering cells, such dyes instead stain the background. (We discuss acidic dyes more in the next lab, which covers negative staining.)

Fixation

Most bacterial staining techniques begin by smearing a small amount of a sample onto a glass slide and then exposing the smear to heat (heat fixing or **heat fixation**) (Fig. 11.4). In some procedures, a chemical reagent may be used as a fixative (**chemical fixation**). Fixation, whether chemical or heat based, adheres the sample to the slide, so it is not as easily washed away during the staining process, and it kills most of the cells in the specimen, making it safer to handle. In simple staining, we typically use heat fixation, which is easily performed by passing the slide through the flame of a Bunsen burner or holding the slide near the opening of a benchtop incinerator (Fig. 11.5).



11.3 Chemistry of Basic Stains • Basic stains have a positively charged chromogen, which readily associates with the negatively charged bacterial cell to colorize it.


1. Add the Sample to the Slide
Liquid sample
If using a liquid sample, place a drop of it onto the slide with a loop. If placing more than one organism on a slide, leave space between each sample. Flame sterilize the loop once finished.
Solid sample
If the sample is growing on solid media, use an inoculating needle or loop to transfer the specimen into a drop of water and gently mix. If placing more than one organism on a slide, leave space between each sample. Flame sterilize the loop once finished.
2. Let the Slide Air-dry
The smear should appear cloudy once dried.
3. Heat Fix the Sample
Using a slide holder, pass the slide (smear side up) through the Bunsen burner flame two or three times.
If using an incinerator, hold the slide near the incinerator's opening for 5–10 seconds.
Note: The smear should face away from the incinerator's opening.
4. Let the Slide Cool
Once the slide is room temperature, you can proceed with the staining procedure.

11.4 Preparing a Bacterial Smear • Most staining procedures start with preparing and heat fixing a bacterial smear. Preparing uniform bacterial smears will make it easier to obtain consistent staining results.

11.5 Heat Fixation Using an Incinerator • To heat fix a slide using an incinerator, hold the slide near the microincinerator's opening (with the smear facing away from the opening) for 5–10 seconds.

Clinical Applications

Bacterial cells are tiny and often clear, which makes them difficult to see on a slide. Therefore, cells are typically stained with a colored dye to make them more visible under the microscope. Stained samples are then observed so that cell morphology, size, and arrangement can be determined. In a medical laboratory, noting morphological features is helpful for bacterial identification and bacterial identity in turn guides treatment options.

MATERIALS

- Lab coat
- Disposable gloves
- Safety glasses
- Clean glass microscope slides
- Basic dyes (at least one of the following):
  - Methylene blue
  - Safranin
  - Crystal violet
- Squirt bottle with water
- Staining tray or a glass beaker to collect dye rinsed off slides
- Bibulous paper (or paper towels)
- Slide holder
- Compound microscope with oil-immersion lens
- Immersion oil
- Lens paper
- Fresh broth or slant cultures of these recommended organisms:
  - *Bacillus cereus* or any *Bacillus* species
  - *Micrococcus luteus*
  - *Neisseria sicca* or *Moraxella catarrhalis*
  - *Rhodospirillum rubrum* or *Aquaspirillum* species
  - *Staphylococcus epidermidis*
  - *Vibrio* species

PROCEDURE

Today you will learn how to prepare a bacterial emulsion (smear) and perform simple stains. Your instructor may supply several different bacterial species or tell you to stain one organism. Whether you stain different bacterial species or not, you can refer to the Instructional Staining Atlas (pp. 131–135) to see the variety of cell morphologies and arrangements in the bacterial world. While performing the staining procedure, wear a lab coat, gloves, and chemical eye protection. You may remove your gloves and eye protection when using the microscope.

1. **Make Your Bacterial Smears:** A bacterial smear (emulsion) is made prior to most staining procedures. Follow the procedural diagram in Figure 11.4 to prepare smears of three organisms on a slide. Be sure not to mix them. At the same time, make a second slide with the remaining three organisms. Emulsions should be about the size of a dime.
2. **Heat Fix the Smears:** Heat fix each slide as illustrated in Figure 11.4. If you are using a bacterial incinerator, hold the slide (with a slide holder) near the opening for a few seconds (Fig. 11.5).
3. **Stain the Smears:** Following the staining procedure illustrated in Figure 11.6, stain your slides with a basic stain (crystal violet, safranin, or methylene blue) for 1 minute.
4. **Observe Your Slides:** Use a light microscope to observe each slide via bright-field microscopy. If you need a refresher on how to get a sample in focus under oil immersion, refer to Exercise 1. Once the specimen is in focus under the oil-immersion lens, make your observations of the bacterium's cell shape and arrangement. Record your observations in the data sheet at the end of this lab.
5. **Dispose of Your Slides:** Dispose of the slides and used stain according to your laboratory's policy. Used slides are typically discarded into a biological sharps container and stain waste is collected into a container for proper chemical removal.

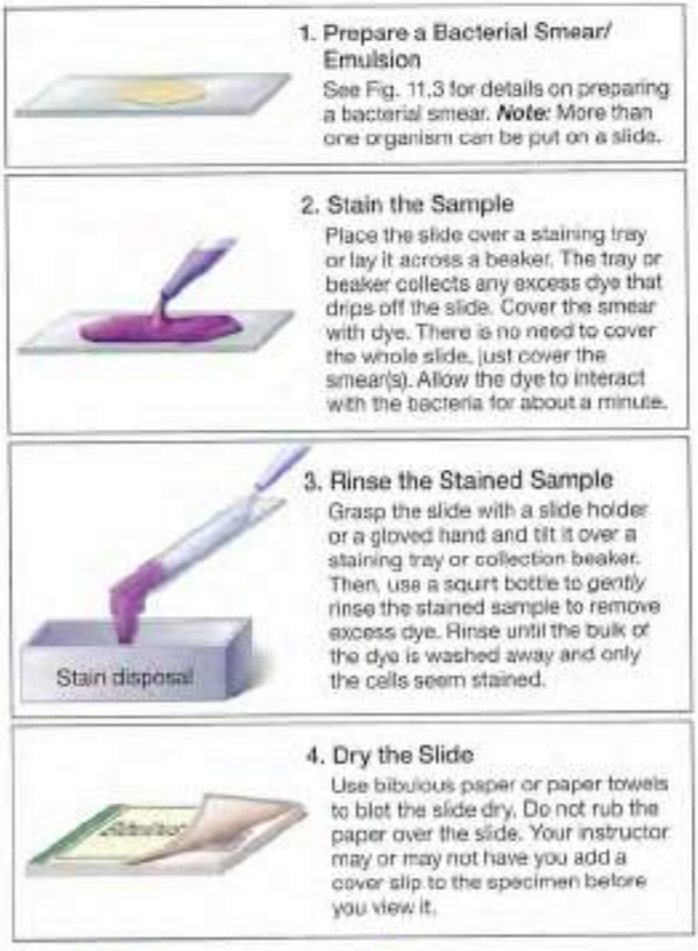

1. Prepare a Bacterial Smear/ Emulsion
See Fig. 11.3 for details on preparing a bacterial smear. Note: More than one organism can be put on a slide.
2. Stain the Sample
Place the slide over a staining tray or lay it across a beaker. The tray or beaker collects any excess dye that drips off the slide. Cover the smear with dye. There is no need to cover the whole slide, just cover the smear(s). Allow the dye to interact with the bacteria for about a minute.
3. Rinse the Stained Sample
Grasp the slide with a slide holder or a gloved hand and tilt it over a staining tray or collection beaker. Then, use a squirt bottle to gently rinse the stained sample to remove excess dye. Rinse until the bulk of the dye is washed away and only the cells seem stained.
Stain disposal
4. Dry the Slide
Use bibulous paper or paper towels to blot the slide dry. Do not rub the paper over the slide. Your instructor may or may not have you add a cover slip to the specimen before you view it.

FIGURE 11.6 Simple Staining Procedure

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 11

DATA SHEET

Simple Stains and Bacterial Cell Morphology

During the Lab

1 Record your observations from the exercise.

Organism Name	Dye Used	Cellular Shape and Arrangement (morphology) (include a written description and a detailed sketch of some representative cells)

Lab Related Questions

1 What can a simple stain reveal? (Select all that apply.)

a. Cell shape
b. Structural features such as the presence of a capsule
c. Cell size
d. Gram property
e. Cellular arrangement

2 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ The simple stain technique employs two or more dyes.

_____ The simple stain technique is performed on live bacteria.

_____ The simple stain technique involves a heat-fixation step.

_____ During simple staining, the bacterium is exposed to the dye for about 10 minutes.

3 Place the following steps of the simple stain in order from first to last; note some steps may not be used at all.

I. Heat fix the smear.
II. Add the chemical fixative.
III. Add the dye.
IV. Make a bacterial smear.
V. Rinse off excess dye.
VI. Blot the slide dry.
VII. Allow the smear to air dry.
VIII. Heat the dye.

Proper order: _____

4 Select the feature of a basic dye (select all that apply).

a. Alkaline
b. Negative charge
c. Stains the interior of cells
d. Includes methylene blue
e. Includes a chromagen

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 11
DATA SHEET
(continued)

5 Assume you are viewing the following bacteria. What shapes and arrangements are represented?

Shape: _____ Arrangement: _____

Shape: _____ Arrangement: _____

Shape: _____ Arrangement: _____

Shape: _____ Arrangement: _____

Shape: _____ Arrangement: _____

Critical Thinking

1 Assume you are staining a bacterial sample, and at the end of the procedure there are barely any stained cells on the slide. Interestingly, your lab partner, who used the same stock bacterial sample as you, as well as the same reagents, sees plenty of cells on their slide. Provide three possible ways you may have performed the staining procedure wrong to obtain the described results.

2 Why would growing a bacterium in liquid culture potentially make it easier for you to determine the bacterium's natural cellular arrangement?

EXERCISE 12

Negative Stains

After doing this exercise, you should be able to:

1. Perform a negative stain of bacterial smears.
2. Describe and identify bacterial shapes and arrangements.
3. List the features of basic versus acidic dyes.
4. Discuss the differences between the simple stain and the negative stain.
5. Perform oil-immersion microscopy to view your own stained bacterial samples.

Background

To avoid confusion, it's worth noting that because the negative staining procedure employs only one dye, it can technically be considered a type of simple staining protocol. That said, most microbiologists use the phrase "simple stain" to refer to staining a sample with a basic dye and reserve the phrase "negative staining" to refer to procedures where a single dye is used to color the background of the sample versus the specimen itself (Fig. 12.1). We follow this tradition and encourage you to also consider these procedures as distinct from one another; they are performed differently and the dyes used are chemically different.

The negative staining technique uses a dye with an acidic chromogen. Recall from Exercise 11 that acidic chromogens carry a negative charge. The negative charge on the bacterial surface repels the negatively charged chromogen, so the cell remains unstained against a colored background (Fig. 12.2).

As with simple staining, the negative stain only reveals basic morphological information about a bacterium, namely cell shape, cellular arrangements, and size. The distinct advantage of a negative stain over the simple stain is the negative stain does *not* include a fixation step (no chemical or heat fixation is applied). Since we do not expose the specimen to harsh chemicals or heat that can distort cell features, the negative staining procedure can reveal a species' truest size, shape, and cellular arrangement. As a case in point, you can compare how *Rhodospirillum rubrum* appears less "plump" in Figure 11.1 as compared to its appearance in Figure 12.1.

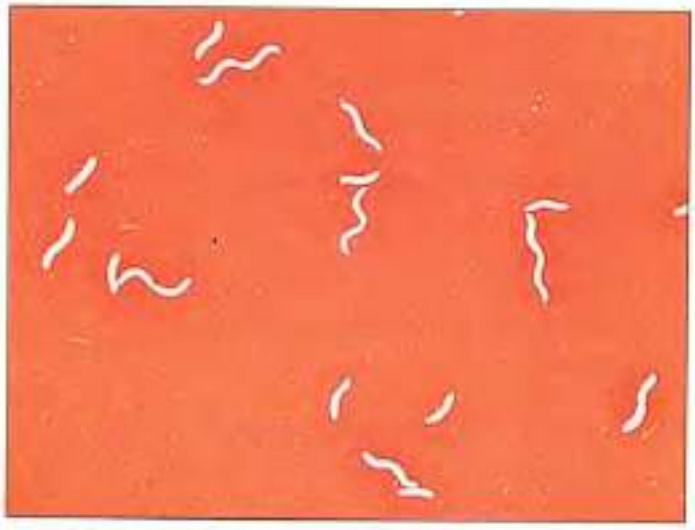


Figure 12.1 Negative Stained Bacterium • This is a negative stain of the spiral-shaped bacterium *Rhodospirillum rubrum*. Notice that the stain is associated with the background versus staining the cells. The organism was grown in a broth culture, stained using Congo red, and viewed with bright-field microscopy. This photograph was taken at 1,000× (oil immersion).

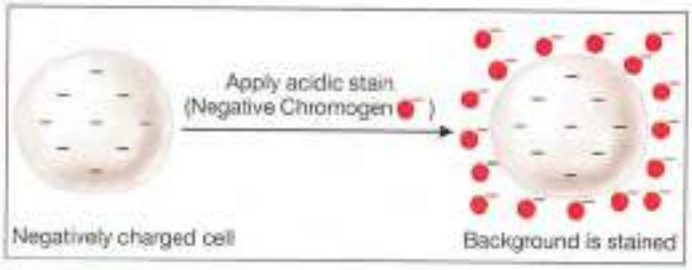


Figure 12.2 Chemistry of Acidic Stains • Acidic stains have a negatively charged chromogen that negatively charged cells repel. Thus, the background is colored and the cell remains transparent.

Clinical Applications

The negative staining technique is used to determine morphology and cellular arrangement in bacteria that are too delicate to withstand heat fixing. A primary example is the spirochete *Treponema pallidum*, which causes syphilis. This bacterium is easily distorted by the heat fixing of other staining techniques. Also, where determining the accurate size is crucial, a negative stain can be used because it produces minimal cell shrinkage.

MATERIALS

- Lab coat
- Disposable gloves
- Safety glasses
- Clean glass microscope slides
- Glass cover slips (as an optional way to drag the dye across the slide)
- Acidic dyes (at least one of the following):
  - Nigrosin
  - India ink
- Paper towels
- Compound microscope with oil-immersion lens
- Immersion oil
- Lens paper
- Fresh broth or slant cultures of these recommended organisms:
  - *Bacillus cereus* or any *Bacillus* species (ideally the same used in Ex. 11)
  - *Micrococcus luteus*
  - *Rhodospirillum rubrum* or *Aquaspirillum* species (ideally the same used in Ex. 11)

PROCEDURE

Today you will perform negative stains on three different organisms. You also will have the opportunity to compare the sizes of *Bacillus cereus*, *Micrococcus luteus*, and *Rhodospirillum rubrum* (or their substitutes) measured with the negative stain to their sizes as determined using a simple stain. You may wish to divide the workload with your lab partners so all specimens are stained by someone within your group. Then, if you don't have time to stain all the specimens yourself, you can examine slides prepared by your lab partner(s). You should refer to the Instructional Staining Atlas (pp. 131–135) to study the variety of cell morphologies and arrangements that bacteria assume; this will help you tackle the Lab Related Questions at the end of this exercise. While performing the staining procedure, wear a lab coat, gloves, and chemical eye protection. You may remove your gloves and eye protection when using the microscope.

1. **Stain your Sample:** Following the staining procedure illustrated in Figure 12.3, stain your slides with an acidic stain (nigrosin or India ink). *Note:* In this procedure, we add only one specimen per slide and there is not a heat-fixation step.
2. **Dispose of the Spreader:** Dispose of the slide or cover slip you used to drag the sample across the slide. A biohazardous sharps container is typically recommended for this.
3. **Observe Your Slides:** Use a light microscope to observe each slide via bright-field microscopy. If you need a refresher on how to get a sample in focus under oil immersion, refer to Exercise 1, page 21. Once the specimen is in focus under the oil-immersion lens, make your observations of the bacterium's cell shape and arrangement. Record your observations in the data sheet at the end of this lab.
4. **Dispose of Your Slides:** Dispose of the slides according to your laboratory's policy. Used slides are typically discarded into a biological sharps container.

1. Add Dye to the Slide

Place the slide on a paper towel on the bench space.

Place a drop of acidic stain (nigrosin in this example) at one end of a clean slide.

2. Add Bacteria to the Slide

With the slide resting on the paper towel-covered bench space, use an inoculating loop to transfer a small amount of bacteria into the drop of dye. Don't over-inoculate and avoid spattering the mixture. Sterilize the loop after emulsifying.

3. Angle a Spreader to Contact the Emulsion

Here we use a second clean slide (or a glass coverslip) as a "spreader." Place the spreader at a roughly 30° angle so it contacts the emulsion.

4. Drag the Emulsion Across the Slide

Using a gentle and smooth motion, drag the spreader across the slide so the emulsion is thinly spread over the slide. This should be one uninterrupted and fluid motion—do not pick up the spreader until the end of the slide is reached or you will have a choppy preparation.

5. Air-dry the Slide

Allow the sample to air-dry—it will appear matte (non-shiny) when fully dry. Once it's dry, observe the sample under the microscope.

Figure 12.3 Negative Staining Procedure

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 12

DATA SHEET

Negative Stains

During the Lab

1 Record your observations from the exercise.

Organism Name	Dye Used	Cellular Shape and Arrangement (morphology) (include a written description and a detailed sketch of some representative cells)

Lab Related Questions

1 What can a negative stain reveal? (Select all that apply.)

a. Cell shape
b. Cellular arrangement
c. Cell size
d. Cell wall features
e. Structural features such as the presence of endospores

2 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ The negative stain technique employs a single dye.

_____ The negative stain technique is performed on heat-fixed bacteria.

_____ The negative stain technique is better than a simple stain for revealing the organism's truest size.

_____ A negative stain is considered a type of differential stain.

3 Place the following steps of the negative stain in order from first to last; note some steps may not be used at all.

I. Cover the smear with dye.
II. Drag the emulsion across the smear.
III. Heat fix the smear.
IV. Warm the dye.
V. Rinse off excess dye.
VI. Add bacteria to the slide.
VII. Add dye to the slide.

Proper order: _____

4 Select the feature(s) of an acidic dye (select all that apply).

a. Alkaline
b. Basic
c. Negatively charged
d. Includes India ink
e. Lacks a chromogen

5 Why doesn't a negative stain colorize the cells in a bacterial smear?

Critical Thinking

1 Compare the sizes of *Bacillus cereus*, *Micrococcus luteus*, and *Rhodospirillum rubrum* cells (or their substitutes) as measured using a basic stain (Ex. 11) and an acidic stain. What might account for any difference(s)?

2 Congo red is a red acidic stain, and methylene blue is a blue basic stain. What should be the result of staining a bacterial smear with a mixture of eosin and methylene blue?

EXERCISE 13

Gram Stain

After doing this exercise, you should be able to:

1. Perform a Gram stain on bacterial smears.
2. Describe and identify bacterial shapes, arrangements, and Gram reactions.
3. Explain how the Gram stain differentiates between Gram-positive and Gram-negative bacteria.
4. Understand the difference between differential, simple, and negative stains.
5. Discuss why clinicians consider the Gram status of an infectious agent when deciding on a drug treatment.
6. Perform oil-immersion microscopy to view your own Gram-stained bacterial samples.

Background

Unlike simple and negative stains, which only reveal information about cell morphology (size, shape, and cellular arrangement), **differential stains** allow us to categorize cells (or differentiate one group from another) based on physical features. The **Gram stain** is the most commonly used differential stain in microbiology; it differentiates between bacterial cells based on cell wall composition. Another common differential stain is the acid-fast stain, which we'll review in the next lab (Ex. 14).

How the Gram Stain Works

Although there are several minor variations of the Gram-stain procedure, chemically they all work the same way, they all affect the bacterial cell wall in a manner that allows us to differentiate between Gram-positive and Gram-negative bacteria. By the end of a Gram-staining procedure, Gram-positive bacteria appear purple while Gram-negative bacteria appear pink/red (Fig. 13.1).

It's important to note that Gram staining does not *make* bacteria Gram positive or Gram negative any more than a pregnancy test makes a person pregnant. Rather, the procedure chemically highlights the natural differences between the cell walls of these bacteria. For example, Gram-negative bacteria have a thin peptidoglycan layer in their cell wall while Gram-positive bacteria have a thick peptidoglycan layer. In addition, Gram-negative bacteria have an outer membrane while Gram-positive bacteria do not. There are other important differences between these two bacterial groups, but electron microscopy and other evidence show the aforementioned features are the most central to the Gram-stain's chemistry. As we progress through the procedure, Gram-positive cells are dyed purple and remain purple the entire time, while Gram-negative cells go through a variety of color states (Fig. 13.2).

The first step in the procedure is to apply **crystal violet**, a basic (or positively charged) dye, to the bacterial smear. Because crystal violet is the first dye added in this double-staining procedure, it is sometimes referred to as the

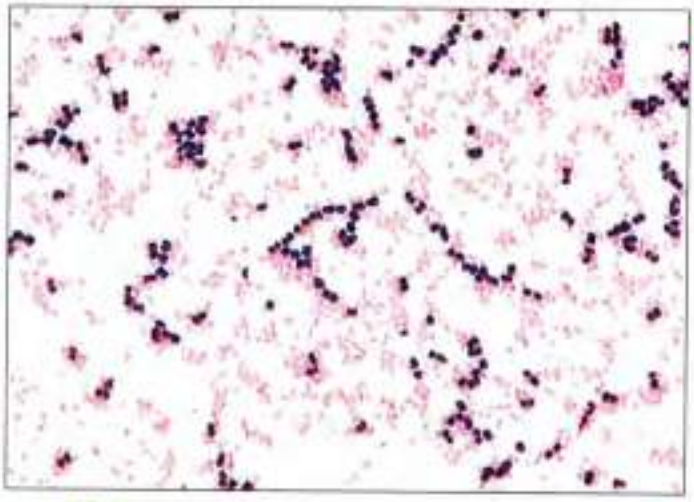


FIGURE 13.1 Gram-Stained *Streptococcus salivarius* and *Erwinia amylovora* • Gram-positive bacteria, such as *S. salivarius*, appear purple at the end of the Gram-stain procedure. Gram-negative bacteria, such as *E. amylovora*, appear pink/red at the end of the procedure.

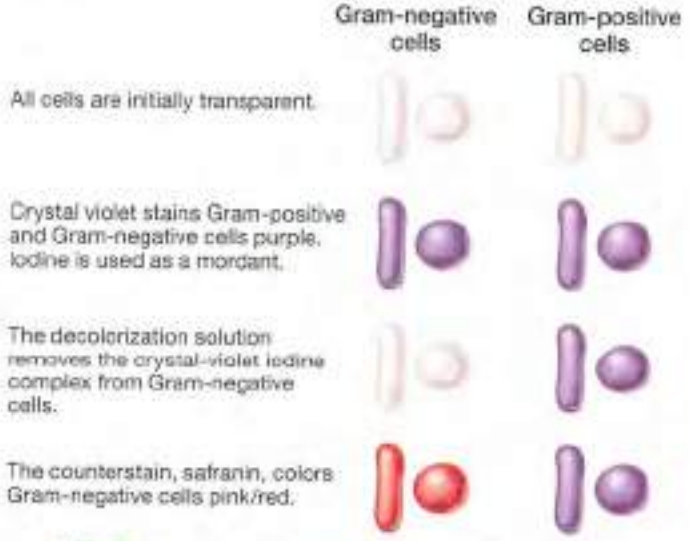

Gram-negative cells
Gram-positive cells
All cells are initially transparent.
Crystal violet stains Gram-positive and Gram-negative cells purple. Iodine is used as a mordant.
The decolorization solution removes the crystal-violet iodine complex from Gram-negative cells.
The counterstain, safranin, colors Gram-negative cells pink/red.

FIGURE 13.2 Gram-Stain Overview • Gram-positive cells remain purple throughout the Gram-staining procedure. Gram-negative cells change colors throughout the procedure, but at the end they appear pink/red.

primary stain. As you likely deduced from its name, crystal violet has a purple color. As such, by the end of the primary staining process, all cells in the sample (regardless of their Gram property) will appear purple. Next, we add Gram iodine, which serves as a **mordant** by bonding to the crystal violet to form a **crystal violet–iodine complex** (or **CV-I complex**). After using water to rinse the excess iodine off the bacterial smear, we add the **decolorizing agent** (usually an acetone-alcohol solution). This is the most critical step in the procedure because it is here where the procedure's chemistry helps us differentiate between Gram-positive and Gram-negative bacteria (Fig. 13.3). After this step, Gram-positive cells remain purple, but the CV-I complex rinses out of Gram-negative cells, rendering them colorless.

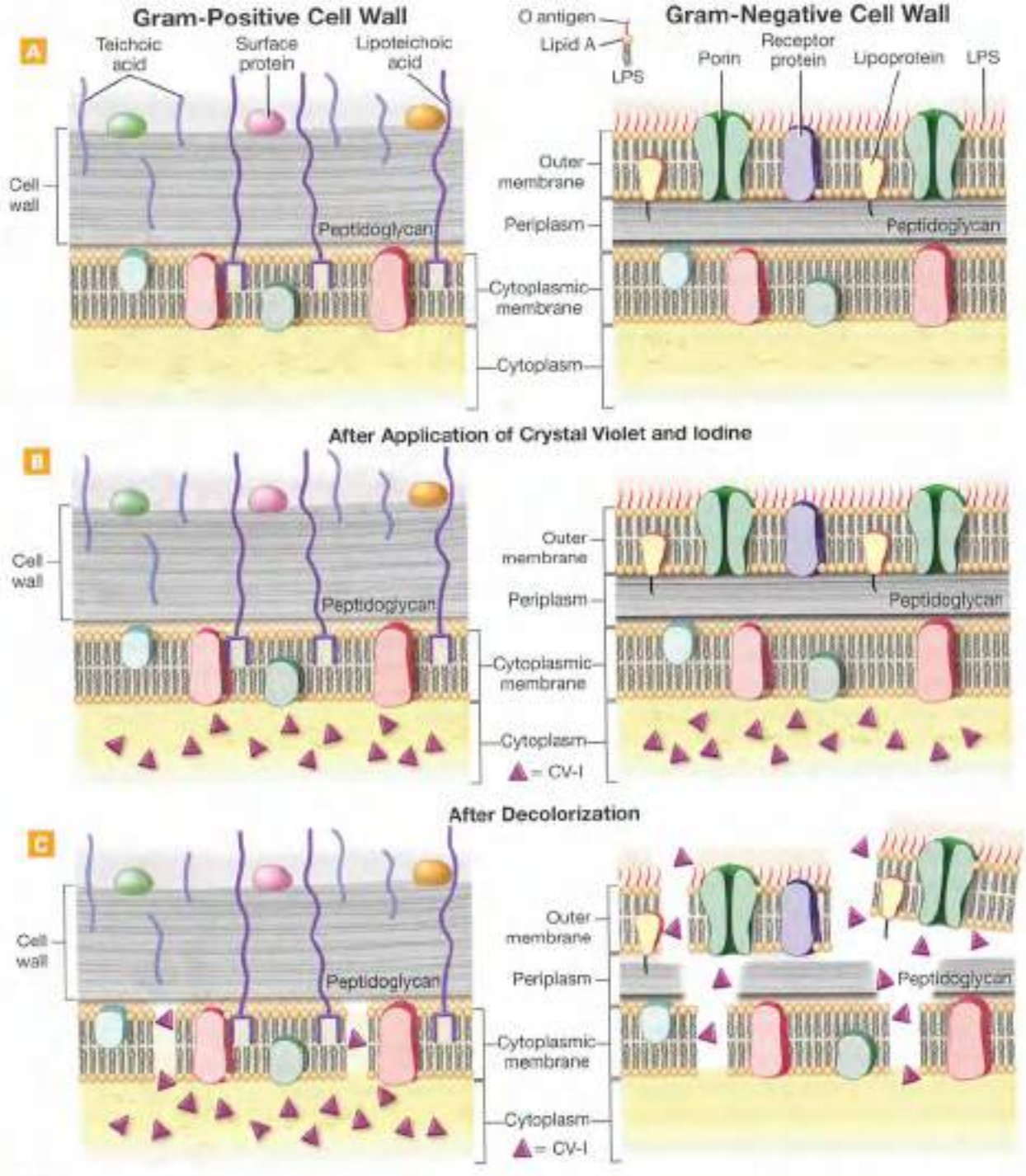


FIGURE 13.3 Chemistry of the Gram Stain • The Gram stain differentiates between Gram-positive and Gram-negative bacteria based on cell wall features. (A) As compared to the Gram-positive bacterial cell wall (**left**), the Gram-negative wall (**right**) has a thin peptidoglycan layer and more lipid (due to the outer membrane). (B) Relatively large crystal violet–iodine complexes (purple triangles) form in the cytoplasm of both Gram-positive and Gram-negative cells. (C) The thicker Gram-positive peptidoglycan layer dehydrates when decolorizer is added, trapping the crystal violet–iodine complexes. The lipid in the Gram-negative cell wall is dissolved by the decolorizer and the thinner peptidoglycan is incapable of preventing extraction of the crystal violet–iodine complex.

The change here is related to the cell wall differences between Gram-positive and Gram-negative cells. The alcohol/acetone in the decolorizer extracts lipids from the Gram-negative cell's outer membrane, which renders the cell wall porous and incapable of retaining the CV-I complex. However, the decolorizer has a different chemical effect on Gram-positive bacteria. In Gram-positive cells, the decolorizer dehydrates the thicker peptidoglycan layer, thereby trapping the CV-I complex inside the cells so the cells remain purple. It's difficult to see colorless cells on glass, so to ensure we don't overlook any of the decolorized Gram-negative cells, we add a second dye called **safranin** as a **counterstain**. This pink/red dye enters the colorless Gram-negative cells so that upon successful completion of the procedure, Gram-negative cells appear reddish-pink.

Gram-Variable Results and Other Considerations

The Gram-staining procedure requires some practice to get right. One source of error is emulsion preparation inconsistency. Remember, a good emulsion is about the size of a dime and dries to a faint haze on the slide. If the bacterial smear is too dense, then you can expect unclear results. As an analogy, consider overstuffing a washing machine. If you pack too many clothes into the machine, then the clothes don't get properly washed because the ratio of clothes to water and detergent is not optimal. Similarly, if you have too many cells in the bacterial smear, they will be layered on top of one another and limit the interaction of the reagents with each of the cells.

A second source of inconsistent Gram-stain results can be the organisms themselves. Some Gram-positive bacteria naturally lose their ability to retain the crystal violet–iodine complex as they age and/or are stressed. *Bacillus* species are especially prone to this effect because they form endospores when stressed. We review endospore formation more in Exercise 16, but suffice it to say here that sporulation (which only certain bacteria can do) damages the bacterial cell wall and can thereby lead to false-negative results (meaning some cells will appear pink, or Gram-negative, when the species is in fact a Gram-positive bacterium). Also, when Gram-positive cell cultures are older, some of the cells will be dying and will therefore have damaged cell walls. If a cell has a damaged cell wall, it won't retain the CV-I complex and will thereby appear pink at the end of Gram staining despite the species technically being Gram positive. To limit such errors, always use cultures that are less than 24 hours old (Fig. 13.4).

Although some organisms like *Bacillus* species may naturally appear Gram variable, most variable results are a consequence of poor technique. The decolorization step is the most crucial step in the procedure and therefore the most likely source of Gram-stain inconsistency. It is possible to **over-decolorize** by leaving the decolorizer on too long and get reddish Gram-*positive* cells (Fig. 13.5). It also is possible to **under-decolorize**, in which case one will see purple Gram-*negative* cells (Fig. 13.6). Neither of these situations changes the actual Gram property of the organism being stained, rather, these are inaccurate results due to poor technique.

In addition to procedural errors and bacterial features like Gram variability, nonbacterial elements can also complicate staining results. For instance, stain crystals from an old or improperly made stain solution can disrupt the field of view (Fig. 13.7) or stain precipitate can be mistaken for bacteria (Fig. 13.8).

Whenever you do a Gram stain it's a good idea to include control smears of known Gram-positive and

FIGURE 13.4 Variable Gram Reaction in *Bacillus* Species • This *Bacillus* species was isolated from aerobic estuarine mud and stained within 24 hours of growing a pure culture. Notice some cells are pink even though *Bacillus* species truly have a Gram-positive cell wall. Because they can form endospores, *Bacillus* species often appear Gram variable.

FIGURE 13.5 Over-Decolorized Gram Stain • This is a direct smear from the gumline. Notice the near absence of any purple cells, a certain indication of over-decolorization since the mouth harbors Gram-positive and Gram-negative bacteria.

Gram-negative organisms alongside the test organism (Fig.13.9). As an alternative control, you can also use a gumline smear, which will naturally include Gram-positive and Gram-negative organisms (Fig. 13.10).

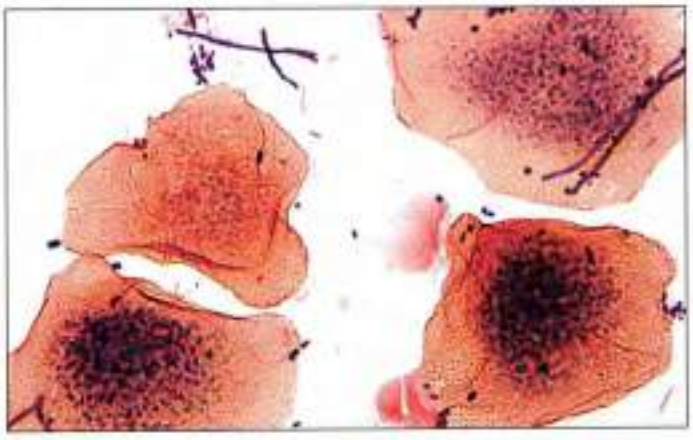


FIGURE 13.6 **Under-Decolorized Gram Stain** • This is a direct smear from the gumline. Notice the purple patches of stain on the epithelial cells. Also notice the variable quality of this stain; the epithelial cell to the left of center stained better than the others.

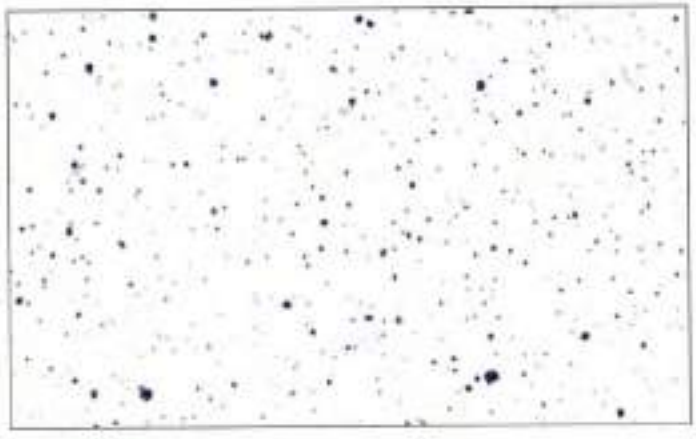


FIGURE 13.8 **Stain Precipitate (Gram Stain)** • If the slide is not rinsed thoroughly or if the stain is allowed to dry on the slide, stain precipitate may form and may be confused with bacterial cells. Their size variability is a clue that they are not bacteria.

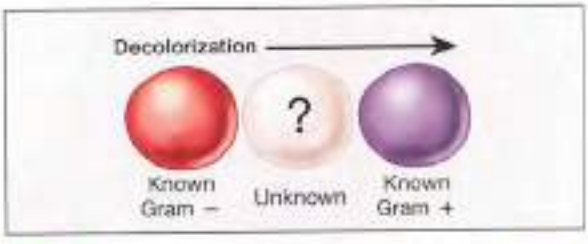


FIGURE 13.9 **Controls to Check Your Gram-Staining Technique** • It's a good idea to include known Gram-positive and Gram-negative organisms as controls on either side of your unknown organism. Try to place the smears as close to one another as possible because spreading them out across the slide makes it difficult to stain and decolorize them equally. It is also beneficial to put the Gram-negative control at the end where you will apply the decolorizer so you can more easily monitor the decolorizing solution runoff and stop decolorizing just as the runoff starts to appear clear.

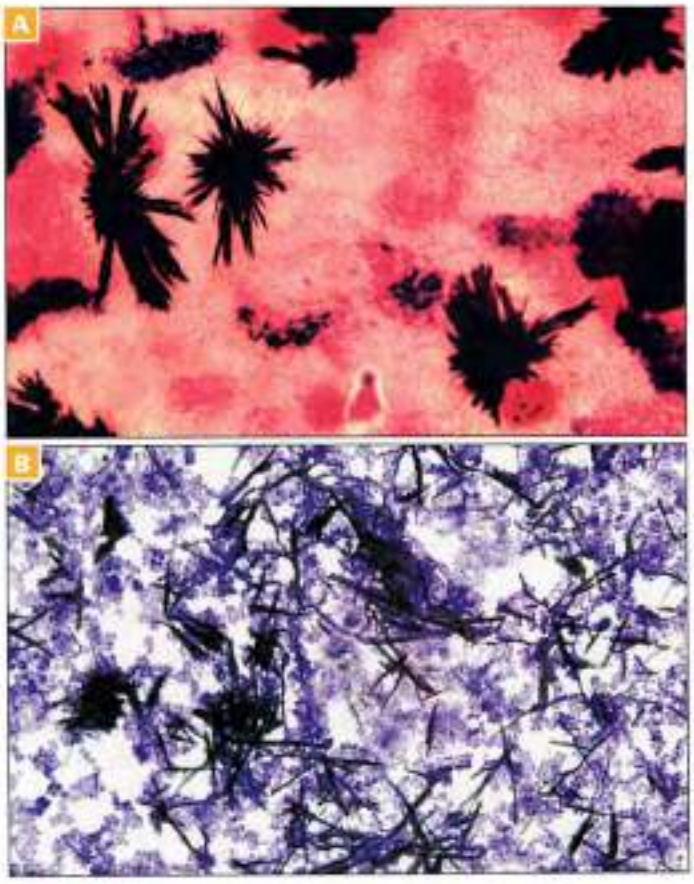


FIGURE 13.7 **Crystal-Violet Crystals (Gram Stain)** • Crystals from two different Gram stains are shown here. (A) This is a gumline direct smear. The bacterial cells are the pink material in the background (this emulsion was far too thick) and the crystals are the large, purple "snowflakes." (B) In this Gram stain of *Micrococcus roseus*, the cells are the lighter purple in the background (can you detect any tetrads?) and the crystals are the darker spikes.

FIGURE 13.10 **A Gumline Smear as a Gram-Stain Control** • A direct smear from the gumline will include Gram-positive bacteria (especially cocci), some Gram-negative cells, and your own nucleated epithelial cells. In this micrograph, different sized Gram-positive cocci predominate, but Gram-negative cells are also present, including two Gram-negative rods (shorter and broader vs. longer and narrower in the two **circles**) and Gram-negative diplococci (**arrow**). Notice that most bacterial cells are on the epithelial cell's surface, which is where they reside in the mouth.

Clinical Applications

The Gram stain, which distinguishes between Gram-positive and Gram-negative bacteria, is the most important and widely used microbiological differential stain. In addition to Gram reaction, this stain also reveals bacterial morphology (shape, size, and arrangement). It is often the first differential test run on a clinical specimen brought into the laboratory for identification. For example, it's an early confirmation tool for diagnosing bacteremia, a bloodstream infection with a mortality rate of about 20% in hospitalized patients. Gram-positive or Gram-negative bacteria can cause bacteremia, but knowing which is the culprit influences the treatment choice. This is because some drugs are more effective against one Gram class of bacteria over another. Many penicillin family drugs are more effective against Gram-positive bacteria than they are against Gram-negative bacteria.

While our drug pharmacopeia includes **broad-spectrum** antimicrobial drugs (agents that are effective against Gram-positive and Gram-negative bacteria), whenever possible it is best to prescribe a **narrow-spectrum** drug that will more specifically target either Gram-positive or Gram-negative bacteria. Any antibiotic can disrupt our normal microbiota, the bacteria that naturally reside in and on our bodies. However, prescribing drugs that more specifically target the infectious agent can reduce normal microbiota disruption and thereby reduce the risks of several undesirable consequences, to include *Clostridioides difficile* (or *C. diff*) diarrhea and **colitis** (inflammation of the colon). Research has shown that a prior antibiotic treatment—especially a broad-spectrum antibiotic therapy—is a key risk factor for developing *C. difficile* colitis. This is because the bacterium is less likely to establish a symptomatic infection when it must compete with the normal microbiota inhabiting the intestinal tract.

MATERIALS

- Lab coat
- Disposable gloves
- Safety glasses
- Compound microscope with oil-immersion lens
- Immersion oil
- Lens paper
- Clean glass microscope slides
- Sterile toothpick
- Squirt bottle with water
- Bibulous paper and/or paper towels
- Staining tray or glass beaker to collect dye waste rinsed off slides
- Slide holder
- Gram-stain solutions (commercial kits are available):
  - Crystal violet
  - Gram iodine solution
  - 95% ethanol (or ethanol/acetone solution)
  - Safranin
- Recommended organisms (overnight cultures grown on agar slants or turbid broth cultures):
  - *Staphylococcus epidermidis* or *Micrococcus luteus*
  - *Escherichia coli* or *Rhodospirillum rubrum*
  - *Neisseria sicca* or *Moraxella catarrhalis*
  - *Corynebacterium xerosis*

3

PROCEDURE

As stated previously (but worth repeating), the Gram stain is the single most important differential stain in microbiology. Therefore, you will probably need to practice it multiple times to get good results. Use the organisms in combinations so you will have one Gram-positive sample and one Gram-negative sample on each slide. Of the listed organisms, *Staphylococcus epidermidis*, *Micrococcus luteus*, and *Corynebacterium xerosis* have the same Gram reaction. Call these "Group A." *Escherichia coli*, *Rhodospirillum rubrum*, *Neisseria sicca*, and *Moraxella catarrhalis* have the other Gram reaction. Call these "Group B." You should place one "Group A" microbe and one "Group B" microbe on each slide along with whatever control your instructor indicates.

While performing the staining procedure, wear a lab coat, gloves, and safety glasses. You may remove your gloves and eye protection when using the microscope.

1. **Make Your Bacterial Smears:** Follow the smear and heat-fixing procedure you used in the simple staining lab (review Fig. 11.4, p. 139, if needed). Place one organism from Group A and one from Group B *immediately next to one another* on the same slide. If you make the emulsions at opposite ends of the slide, you may find it difficult to equally stain and decolorize the smears. Strive to prepare smears of uniform thickness, because thick smears risk being under-decolorized. Also add any control sample that your instructor indicates. Do not exceed three smears per slide. Your instructor may have you prepare only one slide, or several slides with selected Gram-positive and/or Gram-negative bacteria.

2 **Make a Gumline Smear:** (Depending on institutional lab safety regulations, your instructor may not have you prepare a gumline smear.) Wash your hands thoroughly, and then use a sterile toothpick to obtain a sample from your teeth at the gumline. (Do not draw blood! What you want is easy to collect from the gingival pocket, the space between your teeth and gums.) Transfer the sample to a drop of water on a clean glass slide, air dry, and heat fix.

3 **Stain the Smears:** Follow the procedure illustrated in Figure 13.11 to stain your slides. Start by staining the pure cultures you placed on slides in Step 1. Once you have evaluated your technique and find it is consistent, then you may stain the gumline sample you prepared.

If your preparations do not give "correct" results, the most likely source of error is in the decolorization step. When the runoff is clear (generally within 10 seconds, shorter if the acetone concentration is high), *immediately* rinse with distilled H_2O. The decolorizer continues decolorizing until you have rinsed it off with water, so anticipate this and compensate accordingly so you do not over-decolorize.

4 **Observe Your Slides:** Use a light microscope to observe each slide via bright-field microscopy. If you need a refresher on how to get a sample in focus under oil immersion, refer to Exercise 1. Once the specimen is in focus under the oil-immersion lens, make your observations of the bacterium's cell shape, arrangement, and Gram reaction. Record your observations in the data sheet at the end of this lab.

5 **Dispose of Your Slides:** Dispose of the slides and used stain according to your laboratory's policy. Used slides are typically discarded into a biological sharps container and stain waste is collected into a container for proper chemical removal.

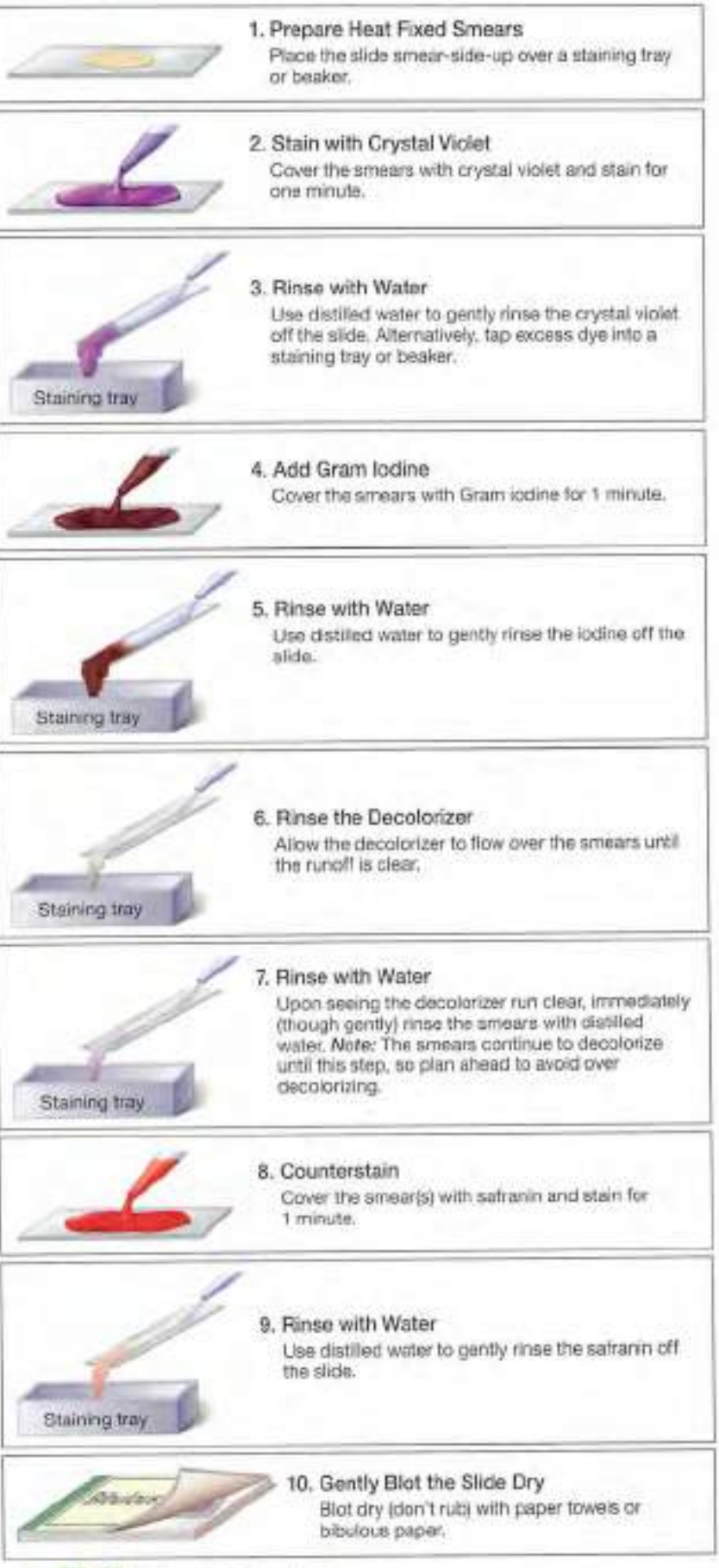

1. Prepare Heat Fixed Smears
Place the slide smear-side-up over a staining tray or beaker.
2. Stain with Crystal Violet
Cover the smears with crystal violet and stain for one minute.
3. Rinse with Water
Use distilled water to gently rinse the crystal violet off the slide. Alternatively, tap excess dye into a staining tray or beaker.
Staining tray
4. Add Gram Iodine
Cover the smears with Gram iodine for 1 minute.
5. Rinse with Water
Use distilled water to gently rinse the iodine off the slide.
Staining tray
6. Rinse the Decolorizer
Allow the decolorizer to flow over the smears until the runoff is clear.
Staining tray
7. Rinse with Water
Upon seeing the decolorizer run clear, immediately (though gently) rinse the smears with distilled water. *Note:* The smears continue to decolorize until this step, so plan ahead to avoid over decolorizing.
Staining tray
8. Counterstain
Cover the smear(s) with safranin and stain for 1 minute.
9. Rinse with Water
Use distilled water to gently rinse the safranin off the slide.
Staining tray
10. Gently Blot the Slide Dry
Blot dry (don't rub) with paper towels or bibulous paper.

FIGURE 13.11 The Gram Stain Procedure

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 13

DATA SHEET

Gram Stain

During the Lab

1 Record your observations from the exercise.

Organism (or source)	Cellular Shape and Arrangement (morphology) (include a written description and a detailed sketch of some representative cells)	Color	Gram Reaction (+/–)

Lab Related Questions

1 The Gram stain is an example of a(n):

a. Simple stain.
b. Negative stain.
c. Acidic stain.
d. Differential stain.
e. Structural stain.

2 What color would Gram-negative bacteria be if you forgot to add the *counterstain* in the Gram-stain procedure?

a. Colorless
b. Purple
c. Pink
d. Green
e. Blue
f. Not enough information

3 What color would Gram-negative bacteria be if you forgot to add the *iodine* in the Gram-stain procedure?

a. Colorless
b. Purple
c. Pink
d. Green
e. Blue
f. Not enough information

4 Some of the following steps are used in the Gram-staining procedure, but some of the listed steps are not used. First, draw a line through the steps you would *not* use. Next, place the remaining steps in sequential order, with "1" being the earliest step.

_____ Heat fix the samples on the slide.

_____ Add crystal violet to the slide.

_____ Add methylene blue to the slide.

_____ Rinse the smears with decolorizer solution.

_____ Add iodine to the smears.

_____ Add bacteria to the slide.

_____ Cover the smears with safranin.

_____ Heat the iodine while on the smears.

5 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ The Gram-staining technique requires one dye.

_____ The Gram-staining technique is best performed on cultures more than 48-hours old.

_____ The Gram-staining technique requires a mordant called iodine.

_____ Gram-positive bacteria should appear purple at the end of the Gram staining procedure.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 13
DATA SHEET
(continued)

6 The Gram stain differentiates between Gram-positive and Gram-negative bacterial cells based on:

a. Cell size.
b. Cell wall differences.
c. Cell shape.
d. Cell density.
e. Cell charge.

7 Why is the Gram stain clinically useful?

8 Assume you perform a Gram stain on a culture you know contains both Gram-positive and Gram-negative bacteria. Upon completing the procedure, all the cells appear pink. What errors may lead to this result? (Select all that apply.)

a. You over-decolorized.
b. You forgot to add iodine.
c. You forgot to heat fix the bacterial smears.
d. You forgot to add the counterstain.
e. You under-decolorized.

Critical Thinking

1 Both crystal violet and safranin are basic stains used to perform simple stains on Gram-positive and Gram-negative cells. This being the case, explain how they end up staining Gram-positive and Gram-negative cells differently in the Gram stain.

2 Your lab partner included Gram-positive and Gram-negative control organisms on their Gram stain of an unknown bacterial species. Their chosen controls were *Escherichia coli* and *Bacillus subtilis*. They try the procedure several times and each time they conclude they are decolorizing too long because both controls end up with mostly pink cells. What might you suggest they try and why?

EXERCISE 14

Acid-Fast Stains

After doing this exercise, you should be able to:

1. Perform an acid-fast stain on bacterial smears.
2. Describe and identify bacterial shapes, arrangements, and acid-fast reactions.
3. Explain how the acid-fast stain differentiates between acid-fast and non-acid-fast bacteria.
4. Understand the difference between the Ziehl-Neelsen and the Kinyoun staining methods.
5. Discuss the clinical significance of the acid-fast stain.
6. Perform oil-immersion microscopy to view your own acid-fast stained bacterial samples.

Background

Like the Gram stain, the acid-fast stain is a differential stain that helps us categorize cells based on cell wall properties. Just as we describe fabrics that resist fading when washed as "colorfast," we can describe cells that resist decolorizing when washed with an acid-alcohol solution as acid-fast. Acid-fast bacteria contain mycolic acid in their cell walls. This waxy substance helps the cells resist decolorization when exposed to an acid-alcohol solution (3% HCl in 95% ethanol). Non-acid-fast bacteria do not have mycolic acid in their cell walls and therefore easily decolorize when rinsed with an acid-alcohol solution (Fig. 14.1). By the end of acid-fast staining, acid-fast bacteria appear red/magenta and non-acid-fast bacteria are the color of the counterstain (usually blue) (Fig. 14.2).

The two main acid-fast staining procedures are the Ziehl-Neelsen (ZN) method (Fig. 14.3) and the Kinyoun (K) method (Fig. 14.4). The choice of method comes down to lab conventions and personal preference. In both methods, the phenolic compound carbolfuchsin is the primary stain because it is lipid soluble and penetrates the waxy cell wall. The main difference between the ZN and K methods is that we use heat in the ZN method to drive the primary stain into cells. Steam heating the preparation softens the waxy mycolic acid layer in acid-fast cells so the carbolfuchsin more readily enters

Acid-Fast
Non-Acid-Fast
Before staining, all the cells are colorless.
Carbolfuchsin, a lipid soluble dye, stains all cells red/magenta regardless of acid-fast property.
An acid-alcohol decolorizing solution, removes the carbolfuchsin from non-acid-fast cells while acid-fast cells retain the stain.
Next, a counterstain (usually methylene blue) is added. Because the counterstain is not lipophilic, it only enters non-acid-fast cells. Therefore, by the end of the procedure, acid-fast bacteria are red/magenta and non-acid-fast cells will appear blue. (Note: Occasionally, brilliant green is used for the counterstain, in which case the non-acid-fast cells would be green).

Figure 14.1 Acid-Fast and Non-Acid-Fast Cell Coloration during the ZN and K Methods • Acid-fast cells stain red/magenta; non-acid-fast cells take on the color of the counterstain, usually blue.

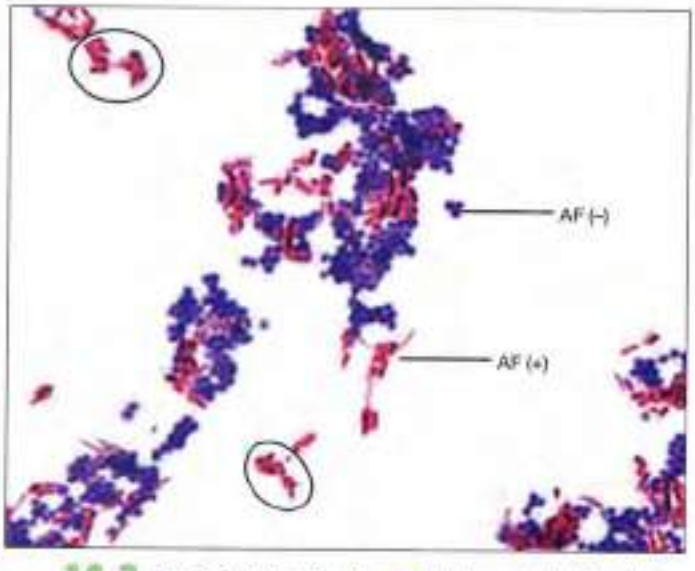


Figure 14.2 Acid-Fast Staining Results • Notice how most of the *Mycobacterium phlei* (acid-fast, or AF+) cells are in clumps; this is an unusual cell arrangement for bacillus-shaped bacteria. They do this because their waxy cell walls make them sticky. The *Staphylococcus epidermidis* cells (non-acid-fast, or AF–) are also in clumps, but that is because they grow as grapelike clusters. Also notice the characteristic beaded appearance of some acid-fast cells (**circled**).

1. Prepare Heat-Fixed Smears
Up to three smears can be added per slide. Place the slide smear-side-up over a beaker of steaming water.

2. Steam the Smears with Carbolfuchsin
Do this step in a well-ventilated area—preferably under a fume hood. Carbolfuchsin is hazardous, so wear gloves and chemical eye protection and avoid inhaling the fumes. Place the slide smear-side-up over a steaming apparatus and then cover the smear(s) with a strip of bibulous paper or paper towel cut slightly smaller than the slide. Apply the ZN carbolfuchsin stain so it soaks the paper. Heat the slide to steaming for 5 minutes (as shown in Fig. 14.5). During the 5 minutes of steaming, keep the paper moist with stain.

3. Rinse with Water
Dispose of the paper with forceps (to prevent stain from getting on your gloves). Then, hold the slide at an angle and use distilled water to gently rinse the carbolfuchsin off the slide. Collect the runoff into a staining tray or collection beaker. Then, if not already there, return to your lab station carrying your slide in a petri plate.

4. Rinse with Decolorizer
Allow the acid-alcohol decolorizer to flow over the smears until the runoff is clear. ***Use increased caution with this reagent.*** Collect the runoff into a collection beaker or staining tray.

5. Rinse with Water
Holding the slide at an angle, gently rinse the smears with distilled water. Catch the runoff in a collection tray or beaker.

6. Counterstain
Cover the smear(s) with methylene blue and stain for 1 minute.

7. Rinse with Water
Use distilled water to gently rinse the methylene blue off the slide. Collect the runoff into a collection beaker or staining tray.

8. Gently Blot the Slide Dry
Blot dry (don't rub) with paper towels or bibulous paper.

FIGURE 14.3 Ziehl-Neelsen (ZN) Acid-Fast Staining Method

1. Prepare Heat-Fixed Smears
Up to three smears can be added per slide. Place the slide smear-side-up over a beaker or staining tray.

2. Stain with Carbolfuchsin (Do Not Heat)
Do this step in a well-ventilated area—preferably under a fume hood. Carbolfuchsin is hazardous, so wear gloves and chemical eye protection and avoid inhaling the fumes. Place the slide smear-side-up over a collection beaker or staining tray. Cover the smears with Kinyoun carbolfuchsin for 5–10 minutes. Do not heat the slide.

3. Rinse with Water
Hold the slide at an angle and use distilled water to gently rinse the carbolfuchsin off the slide. Collect the runoff into a staining tray or collection beaker. Then, if not already there, return to your lab station carrying your slide in a petri plate.

4. Rinse with Decolorizer
Allow the acid-alcohol decolorizer to flow over the smears until the runoff is clear. ***Use increased caution with this reagent.*** Collect the runoff into a collection beaker or staining tray.

5. Rinse with Water
Holding the slide at an angle, gently rinse the smears with distilled water. Catch the runoff into a collection tray or beaker.

6. Counterstain
Cover the smear(s) with methylene blue and stain for 1 minute.

7. Rinse with Water
Use distilled water to gently rinse the methylene blue off the slide. Collect the runoff into a collection beaker or staining tray.

8. Gently Blot the Slide Dry
Blot dry (don't rub) with paper towels or bibulous paper.

FIGURE 14.4 Kinyoun (K) Acid-Fast Staining Method

these typically difficult-to-stain cells (Fig. 14.5). The K method gets around the heating requirement by using a more concentrated carbolfuchsin solution.

A Note About Gram Property and Acid-Fast Bacteria

The waxy wall of acid-fast cells repels typical aqueous stains. As a result, most acid-fast organisms only appear weakly Gram positive when subjected to the Gram-staining procedure. That said, they are still classified as Gram-positive bacteria.

Figure 14.5 Steaming Slides During the Ziehl-Neelsen (ZN) Acid-Fast Staining Procedure • Place your slide (smear-side up) over a beaker of steaming water. Cut a piece of bibulous paper or paper towel so it covers the smears but is smaller than the slide. Add carbolfuchsin to moisten the paper. Keep the paper moist with stain for the entire 5 minutes of steaming. ***Caution!*** Preferably perform this procedure in a fume hood; do not inhale the fumes.

Clinical Applications

The acid-fast stain is an important differential stain used to identify bacteria in the genus *Mycobacterium*, some of which are pathogens (e.g., *M. leprae* and *M. tuberculosis*, causative agents of leprosy and tuberculosis, respectively). Members of the actinomycete genus *Nocardia* (*N. brasiliensis* and *N. asteroides* are opportunistic pathogens) are partially acid-fast. Oocysts of coccidian parasites, such as *Cryptosporidium* and *Isospora*, are also acid-fast. Although several potentially infectious agents are acid-fast, the procedures you perform in this lab are mainly used to identify **acid-fast bacilli (AFB)** as a rapid, preliminary diagnosis of tuberculosis (with greater than 90% predictive value from sputum samples). It also is performed on tuberculosis patient samples to track the progress of antibiotic therapy and determine the degree of contagiousness. A prescribed number of microscopic fields are examined and the number of AFB is determined and reported using a standard scoring system.

MATERIALS

- ☐ Lab coat
- ☐ Disposable gloves
- ☐ Safety glasses
- ☐ Compound microscope with oil-immersion lens
- ☐ Immersion oil
- ☐ Lens paper
- ☐ Clean glass microscope slides
- ☐ Squirt bottle of distilled water
- ☐ Bibulous paper and/or paper towels
- ☐ Staining tray or glass beaker to collect dye waste rinsed off slides
- ☐ Slide holder
- ☐ Sterile wooden sticks, disposable plastic loops, or metal inoculating loops
- ☐ Heating apparatus (beaker of water on a hot plate) for Ziehl-Neelsen procedure only
- ☐ Sheep serum (optional)
- ☐ Acid-fast staining solutions (Ziehl-Neelsen and Kinyoun staining kits are available):
  - ☐ Ziehl-Neelsen staining method
    - Methylene blue stain
    - Ziehl's carbolfuchsin stain
    - Acid alcohol (95% ethanol + 3% HCl)
  - ☐ Kinyoun staining method (complete kits are commercially available)
    - Kinyoun carbolfuchsin
    - Acid alcohol (95% ethanol + 3% HCl)
    - Methylene blue stain, or some kits come with brilliant green as a counterstain
- ☐ Fresh agar slant cultures of these recommended organisms:
  - ☐ *Mycobacterium smegmatis* (BSL-1 strains are available; e.g., ATCC 27203 or ATCC 700084)
  - ☐ *Staphylococcus epidermidis* (BSL-1)

PROCEDURE

Today you will perform an acid-fast stain using the Ziehl-Neelsen (ZN) Method and/or the Kinyoun Method. Both methods are designed to identify members of the genus *Mycobacterium*. While performing the staining procedure(s), wear a lab coat, gloves, and safety glasses. You may remove your gloves and eye protection when using the microscope.

Ziehl-Neelsen (ZN) Method

1. **Make Your Bacterial Smears:** Follow the smear and heat-fixing procedure you used in the simple staining lab (review Fig. 11.4, p. 139, if needed). Make a smear of an acid-fast bacterium and a non-acid-fast bacterium. *Note:* In some labs, sheep serum might be suggested instead of water as the liquid for smear preparation. When preparing the smears, gently but thoroughly mix the *Mycobacterium* smear to reduce cell clumping.
2. **Stain the Smears:** Follow the procedure illustrated in Figure 14.3 to stain your slides. Use a steaming apparatus (such as the one shown in Fig. 14.5) to heat the slide. *Caution!* Do not inhale the carbolfuchsin fumes; ideally perform this procedure in a fume hood or, at the very least, perform this in a well-ventilated space and keep your distance from the fumes.
3. **Observe Your Slides:** Use a light microscope to observe each slide via bright-field microscopy. If you need a refresher on how to get a sample in focus under oil immersion, refer to Exercise 1. Once the specimen is in focus under the oil-immersion lens, make your observations of the bacterium's cell shape, arrangement, and acid-fast reaction. Record your observations in the data sheet at the end of this lab.
4. **Dispose of Your Slides:** Dispose of the slides and used stain according to your laboratory's policy. Used slides are typically discarded into a biological sharps container and stain waste is collected into a container for proper chemical removal.

Kinyoun (K) Method

1. **Make Your Bacterial Smears:** Follow the smear and heat-fixing procedure you used in the simple staining lab (review Fig. 11.4, p. 139, if needed). Make a smear of an acid-fast bacterium and a non-acid-fast bacterium. *Note:* In some labs, sheep serum might be suggested instead of water as the liquid for smear preparation. When preparing the smears, gently but thoroughly mix the *Mycobacterium* smear to reduce cell clumping.
2. **Stain the Smears:** Follow the procedure illustrated in Figure 14.4 to stain your slides. In this procedure you use Kinyoun carbolfuchsin, which is more concentrated than the carbolfuchsin used in the ZN method. Also, we do not heat the smears with the dye in this procedure. *Caution!* Even though we do not heat the carbolfuchsin in this method, it still is a volatile reagent that generates fumes. Do not inhale the carbolfuchsin fumes; ideally perform this procedure in a fume hood or at the very least perform this in a well-ventilated space and keep your distance from the fumes.
3. **Observe Your Slides:** Use a light microscope to observe each slide via bright-field microscopy. If you need a refresher on how to get a sample in focus under oil immersion, refer to Exercise 1. Once the specimen is in focus under the oil-immersion lens, make your observations of the bacterium's cell shape, arrangement, and Gram reaction. Record your observations in the data sheet at the end of this lab.
4. **Dispose of Your Slides:** Dispose of the slides and used stain according to your laboratory's policy. Used slides are typically discarded into a biological sharps container and stain waste is collected into a container for proper chemical removal.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 14

DATA SHEET

Acid-Fast Stains

During the Lab

1 Record your observations from the exercise.

Organism	Staining Method (ZN or K)	Cellular Shape and Arrangement (morphology) (include a written description and a detailed sketch of some representative cells)	Color	Acid-Fast Reaction (+/−)

Lab Related Questions

1 The acid-fast stain is an example of a(n):

a. Simple stain.
b. Negative stain.
c. Acidic stain.
d. Structural stain.
e. Differential stain.

2 What color would non-acid-fast bacteria be if you forgot to add the counterstain in the procedure?

a. Colorless
b. Purple
c. Pink
d. Green
e. Blue
f. Not enough information

3 What color would non-acid-fast bacteria be if you used a green counterstain?

a. Colorless
b. Purple
c. Pink
d. Green
e. Blue
f. Not enough information

4 Some of the following steps are used in the acid-fast Ziehl-Neelsen staining procedure, but some of the listed steps are not used. First, draw a line through the steps you would *not* use. Next, place the remaining steps in sequential order, with "1" being the earliest step.

_____ Heat fix the samples on the slide.

_____ Add bacteria to the slide.

_____ Add methylene blue to the slide.

_____ Steam the smears with carbolfuchsin.

_____ Add acid-alcohol to the slide.

_____ Heat the methylene blue.

5 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ The acid-fast stain helps clinicians diagnose tuberculosis.

_____ The acid-fast staining technique requires two dyes.

_____ The Ziehl-Neelsen acid-fast staining technique can be performed without heat.

_____ Acid-fast bacteria are considered Gram negative.

6 The acid-fast differentiates between acid-fast and non-acid-fast bacterial cells based on:

a. Cell wall differences.
b. Cell size.
c. Cell shape.
d. Cell density.
e. Cell charge.

7 Why is the acid-fast stain clinically useful?

8 The presence of _____ in the bacterial cell wall is what makes a cell acid-fast.

Critical Thinking

1 Assume you put *Mycobacterium smegmatis* and *Staphylococcus aureus* on the same slide and perform the ZN acid-fast staining procedure on the samples. While decolorizing, you realize the clock you are watching is not working properly. Assuming you over-decolorized during the procedure, what would you expect to see as a result? Explain your reasoning.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 14
DATA SHEET
(continued)

2 The mycolic acid layer in acid-fast bacteria is a waxy and somewhat hard to penetrate substance. Knowing this, what assumptions can you make about the timeline for infections caused by acid-fast bacteria and the treatment regimens used to manage such infections?

EXERCISE 15

Capsule Stain

After doing this exercise, you should be able to:

1. Perform a capsule stain on bacterial smears.
2. Describe the structure and function of a bacterial capsule.
3. Explain how the capsule stain works.
4. Understand the clinical significance of the bacterial capsule.
5. Perform oil-immersion microscopy to view your own capsule stained bacterial samples.

Background

The capsule stain is a structural stain that allows us to view bacterial **capsules**, a sticky extracellular polysaccharide or peptide layer that certain bacteria rely on for adhesion and protection. Because bacterial capsules have a neutral charge, they do not readily take up acidic or basic dyes. The capsule stain technique takes advantage of this characteristic by staining *around* the cells with an acidic stain such as Congo red or nigrosin and then staining the cell itself with a basic dye. Provided the bacterial species you stained makes a capsule, the final effect is quite pretty; you'll see a colored slide background dotted with stained cells surrounded by a white halo (the unstained capsule) (Fig. 15.1).

The capsule stain incorporates some aspects of negative staining, namely we stain the background of the slide and we do *not* heat fix the sample. Avoiding heat fixation is essential because applying heat will cause cell shrinkage, which can leave an artifactual white halo around cells that might be interpreted as a capsule. Plus, capsules are fragile, so heating the preparation can distort the structure. In place of heat fixing, cells are emulsified in a drop of serum to promote their adhering to the glass slide. The serum can also enhance the size of the capsule so that it is easier to visualize.

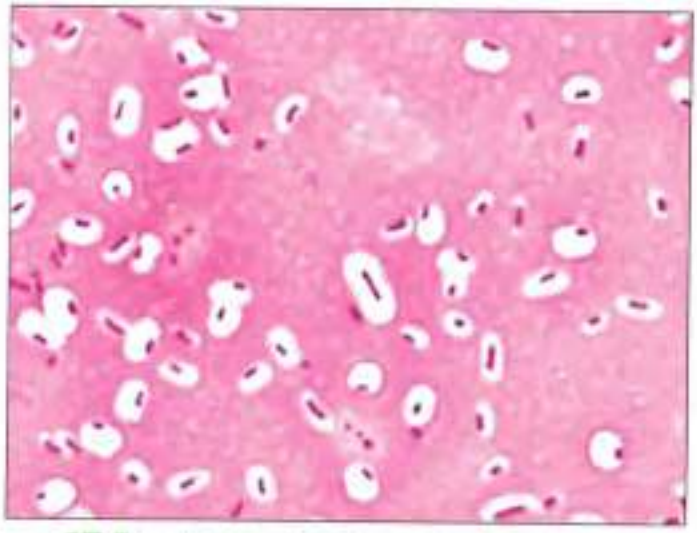


FIGURE 15.1 Capsule Stain • The acidic stain colorizes the background while the basic stain colorizes the cell, leaving the capsules as unstained, white clearings around the cells. Notice the lack of uniform capsule size, and even the absence of a capsule in some cells. There are several variations of the capsule stain. The capsule stain you will perform in this exercise produces different colors than shown in this micrograph, but the principle is the same, capsules appear as white clearings around cells.

Clinical Applications

A number of important Gram-positive and Gram-negative bacterial pathogens make capsules, examples include: *Bacillus anthracis* (anthrax), *Streptococcus pneumoniae* (bacterial pneumonia), *Haemophilus influenzae* (bacteremia, epiglottitis, pneumonia, and meningitis), *Neisseria meningitidis* (meningococcal meningitis), *Klebsiella pneumoniae* (pneumonia), *Salmonella typhi* (typhoid fever), and others. For these bacteria, having a capsule is an important **virulence factor** (a feature that helps an infectious agent cause disease). Not only does having a capsule help a pathogen adhere to surfaces, this extracellular structure also renders bacteria less vulnerable to phagocytosis, an important process that certain white blood cells use to clear invaders from the body. The capsule stain allows us to detect cells capable of producing an extracellular capsule and can therefore reveal information about the bacterium in question.

MATERIALS

- Lab coat
- Disposable gloves
- Safety glasses
- Compound microscope with oil-immersion lens
- Immersion oil
- Lens paper
- Clean glass microscope slides
- Squirt bottle of distilled water
- Bibulous paper and/or paper towels
- Staining tray or glass beaker to collect dye waste rinsed off slides
- Slide holder
- Sterile toothpicks
- Sheep serum (optional)
- Maneval's stain
- Congo red stain
- Coplin staining jar (or a beaker) with distilled water
- Recommended organisms (18- to 24-hour skim milk or tryptic soy agar [TSA] slant pure cultures):
  - *Aeromonas sobria*
  - *Rhizobium leguminosarum*
  - *Klebsiella pneumoniae* (BSL-2) (optional)

PROCEDURE

The capsule stain allows you to visualize an extracellular capsule, if present. Be careful to distinguish between a tiny white halo (a result of cell shrinkage) and a true capsule. While performing the staining procedure, wear a lab coat, gloves, and safety glasses. You may remove your gloves and eye protection when using the microscope.

1. **Stain the Bacteria:** Follow the procedure illustrated in Figure 15.2 to stain your bacteria. Use a separate slide for each specimen. *Note:* Unlike our prior procedures, in this procedure we do not prepare heat-fixed bacterial smears. We do not apply heat at any point in the procedure. Instead we place the bacteria into a drop of sheep serum and dye on the slide and drag it across the slide with a second slide. This procedure is similar to the negative stain procedure in Exercise 12. ***Caution!*** Use BSL-2 precautions if *K. pneumoniae* is stained.
2. **Prepare a Gumline Sample:** Remove your gloves and wash your hands. Then, using a sterile toothpick, obtain a sample from below the gumline in your mouth. (Do not draw blood!) Mix the sample into a drop of water or serum on a slide, and then perform a capsule stain on it.
3. **Observe Your Slides:** Use a light microscope to observe each slide via bright-field microscopy. If you need a refresher on how to get a sample in focus under oil immersion, refer to Exercise 1. Once the specimen is in focus under the oil-immersion lens, make your observations of the bacterium's cell shape, arrangement, and capsule features (if present). Record your observations in the data sheet at the end of this lab.
4. **Dispose of Your Slides:** Dispose of the slides and used stain according to your laboratory's policy. Used slides are typically discarded into a biological sharps container and stain waste is collected into a container for proper chemical removal.

1. Add Dye to the Slide

Place the slide on a paper towel on the bench space. Place a drop of sheep serum at one end of a clean slide. Then, add a drop or loopful of Congo red stain. Be sure to wear gloves.

2. Add Bacteria to the Slide

With the slide resting on the paper towel-covered bench space, use an inoculating loop to transfer a small amount of bacteria into the serum-dye mixture. Don't over-inoculate and avoid spattering the mixture. Sterilize the loop after emulsifying. *Note:* If transferring a BSL-2 organism, use a sterile wooden stick or disposable loop/needle and dispose of it properly when finished.

3. Angle a Spreader to Contact the Emulsion

Here we use a second clean slide (or a glass coverslip) as a "spreader." Place the spreader at a roughly 30° angle so it contacts the emulsion.

4. Drag the Emulsion Across the Slide

Using a gentle and smooth motion, drag the spreader across the slide so the emulsion is thinly spread over the slide. This should be one uninterrupted and fluid motion; do not pick up the spreader until the end of the slide is reached or you will have a choppy preparation. Dispose of the spreader slide in a jar of disinfectant or sharps container.

5. Air-Dry the Slide

Allow the sample to air-dry, do not heat it at all.

6. Add the Second Dye

Place the slide over a staining tray or beaker and cover the spread with Maneval's stain for 1 minute.

7. Rinse Off the Maneval's Stain

Grasp the slide with a slide holder and dip it into a jar of distilled water two or three times to rinse off the Maneval's stain. Change the water in the jar frequently.

8. Remove Excess Water

Gently tap the edge of the slide on a paper towel to remove excess water, then set it down to air-dry. When dry, observe under oil immersion.

15.2 Capsule Stain Procedure

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 15

DATA SHEET

Capsule Stain

During the Lab

1 Record your observations in the provided table. Use a separate line for each organism from your gumline sample that illustrates a capsule or an unusual cell morphology (cell shape and/or arrangement).

Organism	Cellular Shape and Arrangement (morphology) (include a written description and a detailed sketch of some representative cells)	Capsule (+/–)

Lab Related Questions

1 How many dyes are used in the capsule stain?

a. 0
b. 1
c. 2
d. 3
e. 4

2 Choose the true statement(s) about capsules (select all that apply).

a. Capsules appear colorless after a properly performed capsule stain.
b. All bacteria make capsules.
c. Capsules are a virulence factor for some bacteria.
d. Capsules are made of polysaccharides.
e. Capsules are only made by select Gram-positive bacteria.

3 The capsule stain is considered a(n) _____ stain.

a. selective
b. structural
c. basic
d. acidic
e. simple

4 Some of the following steps are used in the capsule staining procedure, but some of the steps are not used. First, draw a line through the steps you would *not* use. Next, place the remaining steps in sequential order, with "1" being the earliest step.

_____ Decolorize the slide.

_____ Add Maneval's stain to the slide.

_____ Add Congo red to the slide.

_____ Add carbolfuchsin to the slide.

_____ Heat fix the samples on the slide.

_____ Add bacteria to the slide.

5 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ The capsule stain requires steaming the dye into the capsule.

_____ The capsule staining technique incorporates aspects of the negative staining technique.

_____ The capsule stain involves an acidic dye and a mordant.

_____ Bacteria that have a capsule are resistant to phagocytosis.

6 Capsules are a(n) _____ structure.

a. intracellular
b. extracellular
c. cell wall
d. cellulose
e. heat-resistant

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 15
DATA SHEET
(continued)

Critical Thinking

1 Some oral bacteria produce an extracellular capsule. Why may a capsule benefit these cells?

2 If having a capsule is an important advantage for certain pathogens, then why wouldn't evolution favor all pathogens being encapsulated?

— Lab Notes —

EXERCISE 16

Endospore Stain

After doing this exercise, you should be able to:

1. Perform an endospore stain on bacterial smears.
2. Describe the structure and function of bacterial endospores.
3. Explain how the endospore stain works.
4. Understand the clinical significance of bacterial endospores.
5. Perform oil-immersion microscopy to view your own endospore stained bacterial samples.

Background

In this lab exercise, you will perform a structural stain called the **Schaeffer-Fulton method** to stain **endospores**, dormant structures that some bacterial species can make in response to harsh environmental conditions (e.g., nutrient depletion, unfavorable temperatures [from boiling to freezing], radiation, high population density, etc.). Because endospores are highly resistant to heat and chemicals (including disinfectants and drugs), the capacity to convert into this dormant form (undergo **sporulation**) allows bacteria to survive adverse conditions for long periods. The record for longevity goes to bacterial spores that were trapped in salt crystals about 250 million years ago and revived in a lab in the year 2000.

Endospores' resistance is due to a combination of factors, including a tough outer covering made of the protein **keratin**, its dehydrated state; DNA protective proteins; and other adaptations. When conditions are suitable again, endospores germinate into metabolically active **vegetative cells**. The keratin in the spore coat also resists staining; therefore, in the Schaeffer-Fulton method (Fig. 16.1), we use steam to drive the primary stain, malachite green, into spores. Alternatively, malachite green can be left on the slide for 15 minutes or more to stain the spores. Malachite green is water soluble and has a low affinity for cellular material, so vegetative cells and **spore mother cells** (which are responsible for producing the endospore) are easily decolorized with water and then counterstained with safranin (Fig. 16.2).

Endospores may be in the middle of the cell (**central**), at the end of the cell (**terminal**), or between the end and middle of the cell (**subterminal**). In some species, endospore location is variable, cells may exhibit a combination of terminal and subterminal, for instance. We also classify endospores by shape—either **spherical** or **elliptical** (**oval**)—and size relative to the cell (that is, whether they cause the mother cell to look swollen or not). Figures 16.3 through 16.5 show the diversity of endospore features.

The genera *Clostridioides*, *Bacillus*, *Clostridium*, *Desulfotomaculum*, *Sporolactobacillus*, *Sporosarcina*, *Thermoactinomyces*, and *Oscillospira* are all examples of endospore-forming bacteria. Among these, *Bacillus*, *Clostridium*, and *Clostridioides* are the clinically important groups. In this exercise, we focus on *Bacillus* as our endospore model. Given enough time, *Bacillus* species will start to make endospores in most media that support their growth. However, we can accelerate sporulation by enriching growth media with manganese because several sporulation enzymes require manganese as a required cofactor. Your instructor may supply you either with cultures grown in sporulating agar or with older cultures not grown on such agar.

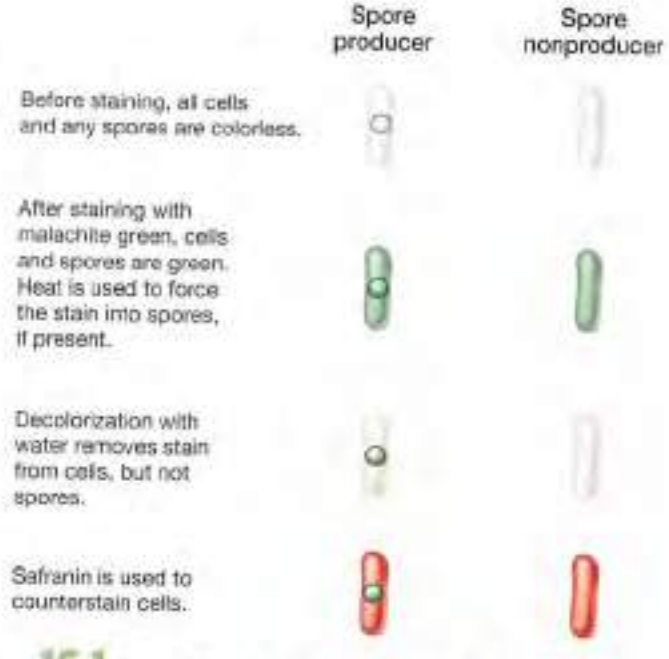

Spore producer
Spore nonproducer
Before staining, all cells and any spores are colorless.
After staining with malachite green, cells and spores are green. Heat is used to force the stain into spores, if present.
Decolorization with water removes stain from cells, but not spores.
Safranin is used to counterstain cells.

Figure 16.1 Schaeffer-Fulton Spore Stain Overview • Upon completion, endospores are green, vegetative and spore mother cells are red.

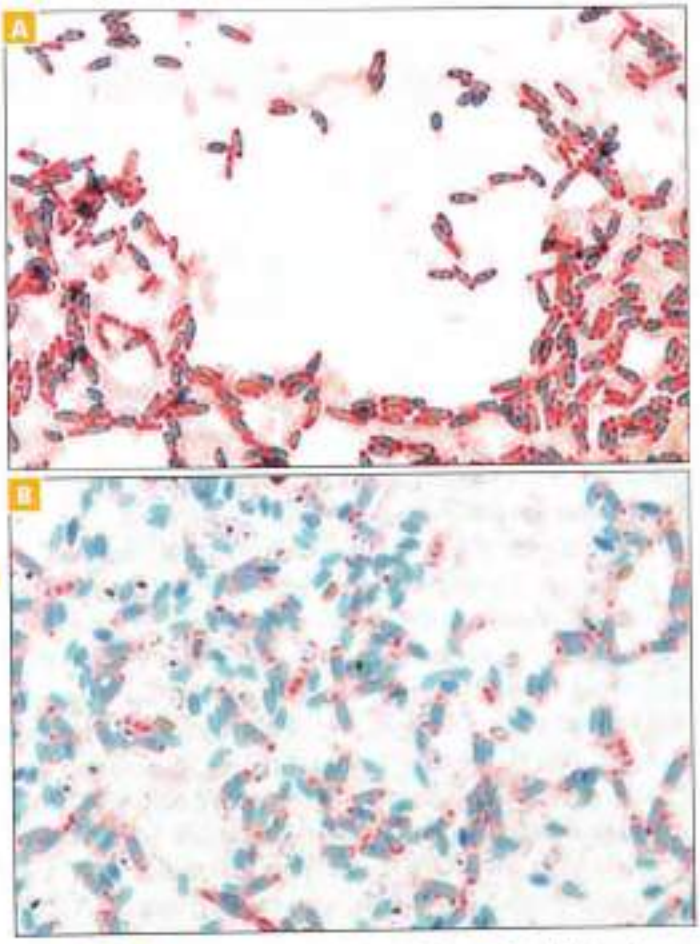


FIGURE 16.2 Culture Age Can Affect Sporulation • Bacteria that can form endospores do so in response to environmental stress, so as nutrients are depleted in older cultures, sporulation is more common. These two cultures illustrate different degrees of sporulation. (A) Most cells in this specimen contain endospores; very few have been released. The light pink rods are probably spore mother cells that have released their spore and have died. (B) This specimen consists mostly of released endospores.

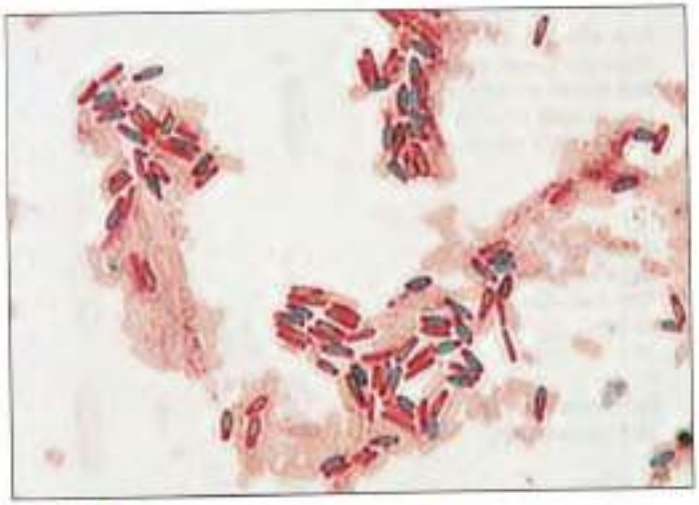


FIGURE 16.3 Central Elliptical Endospores • Most of these *Bacillus megaterium* endospores are centrally positioned, but some are subterminal. All are elliptical, but they do not distend the mother cell.

FIGURE 16.4 Terminal Elliptical Endospores • Shown is *Clostridium tetani* stained by a different endospore stain protocol using carbolfuchsin. Notice how the endospores have caused the ends of the cells to distend (swell).

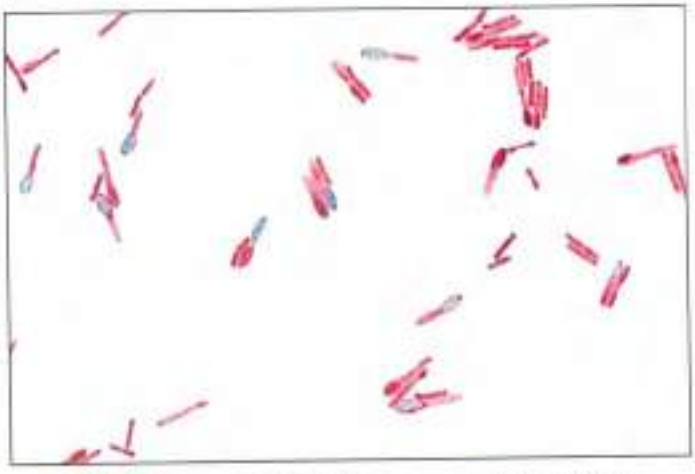


FIGURE 16.5 Subterminal Elliptical Endospores • In these *Clostridium butyricum* cells, most of the green endospores are "capped" by red cytosol, indicating that they are subterminal. Also notice how the spores distend the mother cell, an easily identified characteristic.

A Note on Endospore Function

Students often confuse bacterial spores with fungal spores. It's important to know that unlike fungal spores, bacterial spores do *not* have a reproductive function. A key goal of reproduction is to increase a population. Although the terminology "spore mother cell" may imply a reproductive capacity, endospore formation is not reproductive. Instead, it simply allows the original cell to enter a dormant spore state to weather out harsh conditions and germinate when the environment is more hospitable. The phrase "spore mother cell" is used to emphasize that the cell gives rise to a given spore. Upon spore completion, the mother cell (or rather, what remains of the original cell) is simply a husk that, once destroyed, releases the spore into the environment.

Clinical Applications

The endospore stain is a differential stain used to detect the presence, shape, and location of endospores in bacterial cells. Only a few genera produce spores. Most common are the genera *Bacillus* (more than 100 species) and *Clostridium* (more than 160 species). In addition, several "new" genera formerly classified as *Bacillus* species are also endospore producers. These include *Brevibacillus*, *Geobacillus*, *Paenibacillus*, and *Virgibacillus*, among others.

Most *Bacillus* species are soil, freshwater, or marine **saprophytes** (organisms that live off decaying organic matter), but two are pathogens. *B. anthracis* causes anthrax, and *B. cereus* causes two kinds of food poisoning: emetic and diarrheal. Most *Clostridium* members are harmless soil or aquatic saprophytes; some are notorious pathogens—namely, *C. tetani* (tetanus), *C. botulinum* (botulism), and *C. perfringens* (gas gangrene).

Because endospores are especially resistant to chemicals, heat, and other environmental stresses, they are quite challenging to destroy both in patients and in clinical environments. For example, the pathogen *Clostridioides difficile* (formerly *Clostridium difficile*), which causes infectious diarrhea, can enter the dormant endospore state in response to drug therapies. Thus, it is common for patients who successfully recover from a bout of *C. difficile* diarrhea to relapse within weeks as the spores germinate and reenter an actively growing vegetative state.

MATERIALS

- Lab coat
- Disposable gloves
- Safety glasses
- Compound microscope with oil-immersion lens
- Immersion oil
- Lens paper
- Clean glass microscope slides
- Squirt bottle of distilled water
- Bibulous paper and/or paper towels
- Staining tray or glass beaker to collect dye waste rinsed off slides
- Heating apparatus (beaker of water on a hot plate)
- Slide holder
- Malachite green stain
- Safranin stain
- Recommended organisms:
  - 24-hour and 48-hour sporulating agar slant pure cultures of *Bacillus cereus*
  - 24-hour and 48-hour sporulating agar slant pure cultures of *Bacillus coagulans*
  - 24-hour and 48-hour sporulating agar slant pure cultures of *Bacillus megaterium*
  - 24-hour and 48-hour sporulating agar slant pure cultures of *Bacillus subtilis*

Note: Tryptic soy agar can be substituted for sporulating agar, but incubation times should be adjusted to 48 hours and 5 days.

Recipe for Sporulating Agar

Pancreatic digest of gelatin	6.0 g
Pancreatic digest of casein ($C_{36}H_{57}N_9O_9$)	4.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Dextrose (glucose) ($C_6H_{12}O_6$)	1.0 g
Agar	15.0 g
Manganous sulfate ($MnSO_4$)	0.3 g
Distilled or deionized water	1.0 L

Final pH 6.6 ± 0.2 at 25°C

PROCEDURE

Today you will visualize endospores of as many as four *Bacillus* species. To do this, you will use the Schaeffer-Fulton endospore staining method. In addition, you will be supplied with two cultures of different ages of each species to see if there is a relationship between culture age and endospore production. While performing the staining procedure, wear a lab coat, gloves, and safety glasses. You may remove your gloves and eye protection when using the microscope.

1. **Prepare the Bacterial Smears:** For each organism, prepare and heat fix two smears on the same slide. One smear should be the 24-hour culture, and the other the 48-hour culture (or 48-hour and 5-day cultures if TSA is the medium). Divide the work of slide preparation within your lab group. Minimally, each student should prepare one slide with smears of the 24-hour and 48-hour cultures of one species. ***Caution!*** Use BSL-2 precautions if you have subcultured soil isolates for endospore staining.

2 **Stain the Bacteria:** Follow the procedure illustrated in Figure 16.6 to stain your bacteria. Use a steaming apparatus like the one shown in Figure 16.7. Perform the steaming step in a well-ventilated area (preferably inside a fume hood). Always avoid inhaling the fumes. If you must carry the slide to and from the steaming apparatus, put it in a covered petri plate.

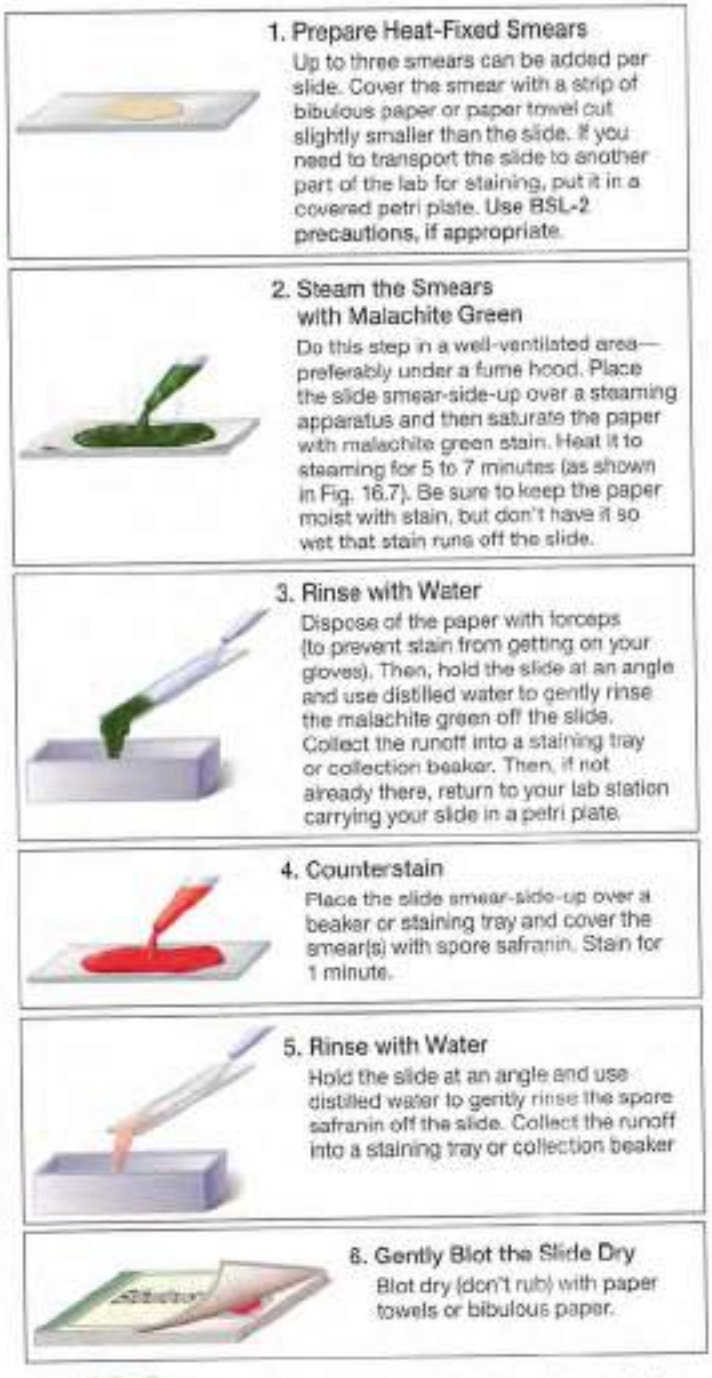

1. Prepare Heat-Fixed Smears
Up to three smears can be added per slide. Cover the smear with a strip of bibulous paper or paper towel cut slightly smaller than the slide. If you need to transport the slide to another part of the lab for staining, put it in a covered petri plate. Use BSL-2 precautions, if appropriate.
2. Steam the Smears with Malachite Green
Do this step in a well-ventilated area—preferably under a fume hood. Place the slide smear-side-up over a steaming apparatus and then saturate the paper with malachite green stain. Heat it to steaming for 5 to 7 minutes (as shown in Fig. 16.7). Be sure to keep the paper moist with stain, but don't have it so wet that stain runs off the slide.
3. Rinse with Water
Dispose of the paper with forceps (to prevent stain from getting on your gloves). Then, hold the slide at an angle and use distilled water to gently rinse the malachite green off the slide. Collect the runoff into a staining tray or collection beaker. Then, if not already there, return to your lab station carrying your slide in a petri plate.
4. Counterstain
Place the slide smear-side-up over a beaker or staining tray and cover the smear(s) with spore safranin. Stain for 1 minute.
5. Rinse with Water
Hold the slide at an angle and use distilled water to gently rinse the spore safranin off the slide. Collect the runoff into a staining tray or collection beaker.
6. Gently Blot the Slide Dry
Blot dry (don't rub) with paper towels or bibulous paper.

FIGURE **16.6** **Schaeffer-Fulton Endospore Staining Procedure** • Perform this procedure with adequate ventilation (preferably in a fume hood), eye protection, a lab coat, and gloves. ***Caution!*** Employ BSL-2 precautions if you have subcultured a soil isolate for endospore staining.

3 **Observe Your Slides:** Use a light microscope to observe each slide via bright-field microscopy. If you need a refresher on how to get a sample in focus under oil immersion, refer to Exercise 1. Once the specimen is in focus under the oil-immersion lens, make your observations of visible endospores for each species. Be sure to note their shapes, locations, and relative sizes.

4 **Dispose of Your Slides:** Dispose of the slides and used stain according to your laboratory's policy. Used slides are typically discarded into a biological sharps container and stain waste is collected into a container for proper chemical removal.

FIGURE **16.7** **Steaming the Slide During the Endospore Stain** • Carefully steam the slide to force the malachite green into the endospores. Do not boil the slide or let it dry out. Keep it moist with stain for up to 7 minutes of steaming. ***Caution!*** This should be performed in a well-ventilated area (preferably in a fume hood) while wearing a lab coat, safety glasses, and gloves.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 16

DATA SHEET

Endospore Stain

During the Lab

1 Record your observations in the provided table. Use a separate line for each bacterial smear you observed.

Organism (include culture age)	Cellular Shape and Arrangement (morphology) (include a written description and a detailed sketch of some representative cells and spores)	Spores (present or absent)	Spore Shape and Position (if present)

Lab Related Questions

1 What is the primary stain in the Schaeffer-Fulton endospore staining method?

a. Methylene blue
b. Malachite green
c. Safranin
d. Crystal violet
e. Carbolfuchsin

2 Choose the true statement(s) about endospores (select all that apply).

a. Endospores resist staining.
b. Endospores stain pink in the endospore staining procedure.
c. *Bacillus* species make endospores.
d. Endospores are dormant structures.
e. Endospores are heat sensitive.

3 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ An endospore's heat resistance is in part due to its low water content.

_____ Endospores serve as reproductive structures.

_____ Endospores are a clinical challenge because they can resist drug treatments.

_____ Adding magnesium to growth media can encourage spore-forming bacteria to make endospores.

4 The Schaeffer-Fulton endospore staining method is considered a(n) _____ stain.

a. acidic
b. basic
c. selective
d. simple
e. structural

5 Some of the following steps are used in the Schaeffer-Fulton endospore staining method, but some of the steps are not used. First, draw a line through the steps you would *not* use. Next, place the remaining steps in sequential order, with "1" being the earliest step.

_____ Rinse the primary stain off the slide with water.

_____ Steam the slide with the primary dye for 5–7 minutes.

_____ Decolorize the slide with acid-alcohol.

_____ Add safranin dye to the slide.

_____ Add Maneval's stain to the slide.

_____ Heat fix the samples on the slide.

_____ Rinse the counterstain off the slide with water.

_____ Blot the slide dry.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 16
DATA SHEET
(continued)

6 The process of endospore formation is called _____.

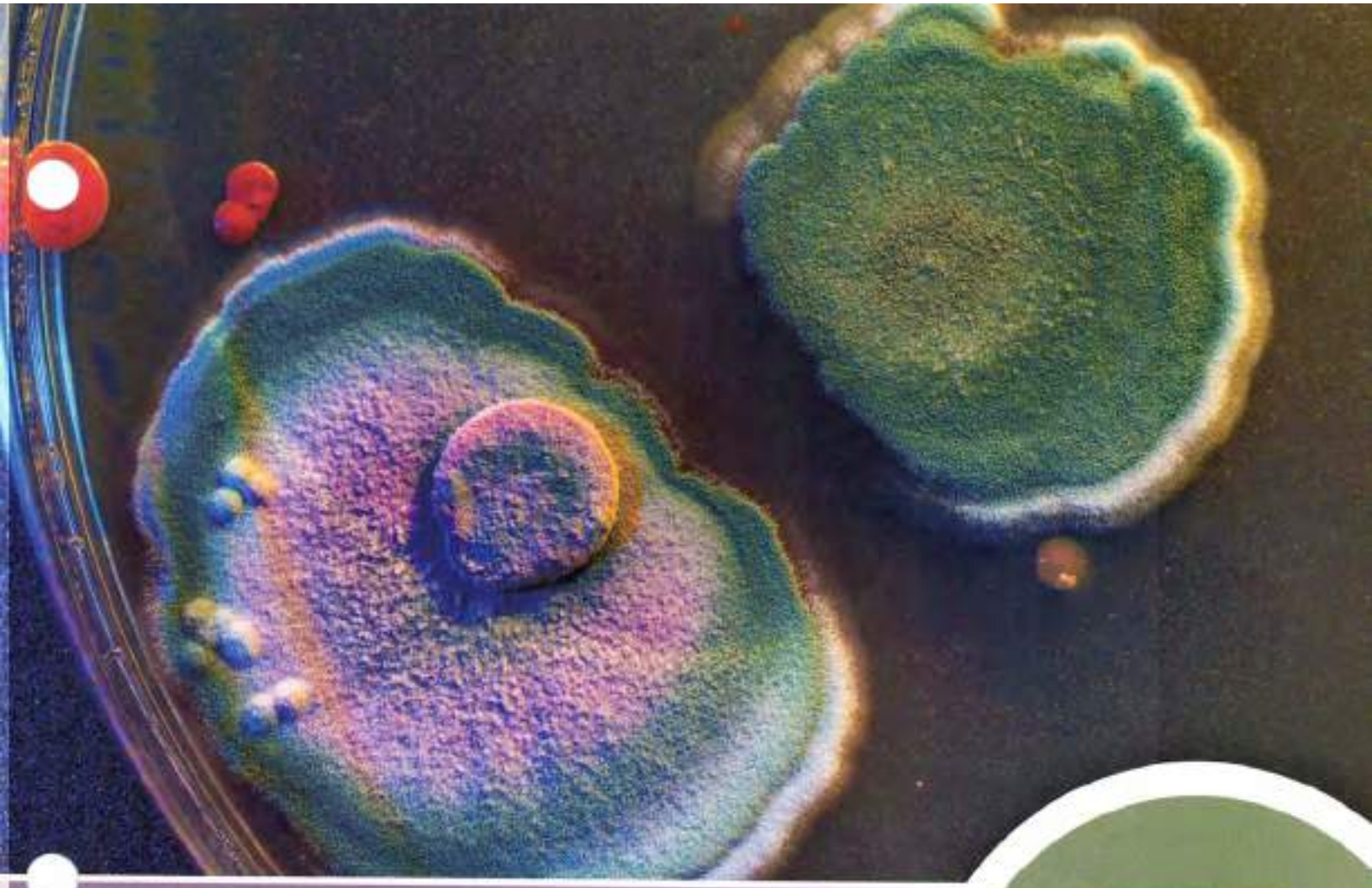
7 Endospores will appear _____ while mother cells appear _____ at the end of the Schaeffer-Fulton endospore staining method.

a. green; blue
b. blue; green
c. green; red
d. red; green
e. green; violet

Critical Thinking

1 Design an endospore staining procedure where carbolfuchsin and methylene blue are used as the dyes. Describe your protocol based on the fundamentals of the Schaeffer-Fulton endospore staining method.

2 Some people harbor *Clostridioides difficile* as a natural inhabitant of their gastrointestinal tract. However, this bacterium is unlikely to cause diarrhea unless the person recently underwent an antibiotic regimen (especially a broad-spectrum antibiotic treatment). Why would taking a broad-spectrum antibiotic treatment put a patient at risk for *C. difficile* diarrhea?



SECTION

4

Media Properties and Conditions that Affect Bacterial Growth

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— Lab Notes —

EXERCISE 17

Evaluating Complex Versus Defined Media

After doing this exercise, you should be able to:

1. Differentiate between fastidious and non-fastidious bacteria.
2. Define synthetic versus complex media and describe how they are different.
3. Compare chemotrophs to phototrophs.
4. Compare heterotrophs to autotrophs.
5. Apply aseptic bacterial transfer and culture techniques.

Background

All organisms need an energy source and a carbon source. Regarding energy sources, we can classify organisms as either **chemotrophs** or **phototrophs**. Chemotrophs get their energy by breaking down nutrients, while phototrophs rely on solar energy. In terms of carbon sources, we classify organisms as either **autotrophs** ("self-feeding" organisms that convert inorganic carbon such as CO_2 into organic carbon) or **heterotrophs** (organisms that obtain their carbon from organic nutrients). Putting these categories together, plants are **photoautotrophs** while we (and most clinically important microorganisms) are **chemoheterotrophs**.

Some heterotrophs are metabolically flexible and can build most of their organic compounds from scratch. Such microbes only need a few simple organic compounds to grow and divide, which is why we classify them as **non-fastidious**. In contrast, **fastidious** microbes are less self-sufficient; they can't build their necessary organic compounds from scratch and instead they obtain those compounds from their environment. This means fastidious microbes usually require an enriched environment to grow and divide, while non-fastidious microbes can survive on a few simple organic nutrients. In general, autotrophs are the least fastidious and therefore the most self-reliant in terms of nutrients, even as compared to the most non-fastidious heterotrophs.

To successfully grow a microbe in the laboratory, we must satisfy its nutritional needs. The absence of even one required nutrient can prevent growth. The more fastidious the organism, the more ingredients a medium typically must have. **Complex media** (also called **enriched media**) contain extracts from, or digests of, plant and/or animal matter and are usually rich in nutrients. Because complex media include less precisely defined organic nutrients, one batch may vary from another. For example, the amount of glucose in the blood added to one batch of blood agar may vary from the blood used to make another batch of such agar. Complex media are useful for growing the greatest variety of culturable microbes and are therefore commonly used in clinical microbiology labs to assess clinical samples.

In contrast, **defined media** (sometimes called **synthetic media**) get their name from the fact that each organic and inorganic ingredient is precisely known and quantified, which means these media are accurately reproducible from batch to batch. As a result, defined media usually grow a narrower range of organisms, usually a specific known organism with well-characterized nutritional needs.

Regardless of a media's classification as complex or defined, we can prepare it in solid or liquid formats; that is, we can find any of these media poured as broths (which is what we'll use in this lab exercise), or as plated and slant formats. To make plated or slant formats, we would add **agar** (a solidifying agent extracted from red seaweed), to broth media. Because most clinically important bacteria do not digest agar, it's a good solidifying agent for clinical microbiology applications.

A Note on Growth Rates

In this exercise, you will assess the relative growth rates of different bacteria grown in different media. In general, all organisms increase their population the fastest when grown under optimal growth conditions. By comparing the turbidity (cloudiness) of each culture, you can determine how each media supported (or failed to support) the growth of a given microbe. By comparing how well each species grew in enriched versus defined media, you can rank the bacteria by their relative fastidiousness.

The term *growth rate*, as used in this manual, refers to reproductive rate. While inherent growth rates can differ from one species to another, we can legitimately compare growth rates of the same organism in different media. However, growth rate comparisons between *different* organisms in the *same* medium require us to assume the inherent growth rates are the same

(or similar). In this exercise we will make this assumption, with the understanding that it may not be true.

Clinical Applications

You've probably had a sample, such as a throat swab, sent to a clinical microbiology lab for a definitive diagnosis. Upon receiving that sample, a clinical lab scientist would have streaked it onto a complex media called blood agar and then checked the growth characteristics the next day to learn about the bacteria present in the sample. A bacterium's growth profile on different media helps microbiologists determine the pathogen's identity. A variety of studies suggest most bacteria are **noncultivable in vitro** (not growable in the lab setting outside of an animal model). A best guess is that we can only grow perhaps 2% of bacterial species in the environment using existing media. When we consider body sites such as the mouth, it's estimated that using currently available methods, we can only grow perhaps half of the species present. Until 2018, *Treponema pallidum* (the causative agent of the sexually transmitted infection syphilis) was not cultivable outside of an animal model. Ultimately, if we are to understand how to more effectively treat bacterial pathogens, we must be able to study them in the laboratory, and understanding what they need to grow is an important step toward that process.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Sterile transfer pipettes (4)
- ❑ Tubes (5 each) of:
  - ☐ Brain-heart infusion broth (BHIB)
  - ☐ Glucose salts broth (GSB)
  - ☐ Nutrient broth (NB)
- ❑ Fresh broth cultures of these four recommended bacteria:
  - ☐ *Escherichia coli*
  - ☐ *Lactococcus lactis*
  - ☐ *Moraxella catarrhalis* (some strains are BSL-2)
  - ☐ *Staphylococcus epidermidis*

Medium Recipes

Nutrient Broth (NB)

Beef extract	3.0 g
Peptone*	5.0 g
Distilled or deionized water	1.0 L

*A protein extract typically made by the breakdown of milk, animal tissue, plants, or microbial cultures (e.g., yeast).

Brain-Heart Infusion Broth (BHIB)

Calf brains, infusion from 200 g	7.7 g
Beef heart, infusion from 250 g	9.8 g
Proteose peptone	10.0 g
Dextrose (glucose) ($C_6H_{12}O_6$)	2.0 g
Sodium chloride (NaCl)	5.0 g
Disodium phosphate (Na_2HPO_4)	2.5 g
Distilled or deionized water	1.0 L

Glucose Salts Broth (GSB)

Glucose ($C_6H_{12}O_6$)	5.0 g
Sodium chloride (NaCl)	5.0 g
Magnesium sulfate ($MgSO_4$)	0.2 g
Ammonium dihydrogen phosphate ($NH_4H_2PO_4$)	1.0 g
Dipotassium phosphate (K_2HPO_4)	1.0 g
Distilled or deionized water	1.0 L

PROCEDURE

In this exercise, you will examine how media composition affects what organism(s) can grow. You will also assess the relative fastidiousness of different bacteria. While performing the lab, wear a lab coat, gloves, and safety glasses.

1. **Obtain Your Media:** Working with your group, obtain five tubes of each medium (a total of 15 tubes). Label each tube with your group name, the date, and the medium's name.

2 **Designating the Tubes for Certain Bacteria:** Divide your 15 tubes of media into five sets; each set should contain the following: one tube of nutrient broth (NB), one tube of brain-heart infusion broth (BHIB), and one tube of glucose salts broth (GSB). Next, label each media set with a different organism name (*E. coli*, *L. lactis*, *M. catarrhalis*, and *S. epidermidis*); label the last set of media tubes "uninoculated."

3 **Inoculate Your Media with the Designated Bacteria:** Mix each stock bacterial tube thoroughly, but be careful not to splash into the cap. *Caution!* Use BSL-2 precautions when transferring *Moraxella catarrhalis*. Use a sterile transfer pipette to transfer 1 drop of the given organism into each type of media (e.g., add 1 drop of *S. epidermidis* into the NB media, 1 drop into the BHIB media, and 1 drop into the GSB media); repeat this process until each organism has been inoculated into each medium. Instead of repeatedly going into the stock sample tube, which will increase the chances of contamination, you may fill the pipette with about 1 mL of broth and deliver the drops with the same pipette to each tube without flaming them. In your group, leave one of each tube uninoculated.

CAUTION!

- When you go to inoculate the media, insert the pipette well into the tube (but avoid touching the glass or the medium).
- Be careful not to spatter as you deliver the drop(s) to each tube, or accidentally drip organism between tubes. Clean up the spill if you do.
- If you don't have enough organism left in the pipette to inoculate a tube, get more organism. You must not empty ("blow-out") the pipette because you will create aerosols.
- Properly dispose of the pipette when finished.

4 **Incubate the Samples:** Incubate all tubes (including the uninoculated ones) at 35 ± 2°C for 24–48 hours.

5 **Clean up:** Save or dispose of the original cultures as directed by your instructor.

→ Next Lab Session

1 **Obtain Your Samples:** Collect your cultures from the incubator (or wherever your instructor placed your samples) and organize your tubes into groups by organism in one direction and by medium in the other.

2 **Mix and Observe the Samples:** Mix all of your tubes well and examine them for turbidity (cloudiness). Avoid splashing broth into the caps. Score relative amounts of growth using "0" for no growth, and "1," "2," and "3" for successively greater degrees of growth. (*Note:* Increased cloudiness or turbidity reflects greater growth.) Choose the tube (regardless of medium) with the most turbidity to act as a "3." And use the uninoculated tubes as your "0" reference point.

3 **Record your results:** Note all observations and results on the data sheet at the end of this lab. (*Note:* Results may vary. Record what you see, not what you expect or what other groups are getting for results.)

4 **Clean up:** Dispose of your samples as your instructor directs.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 17

DATA SHEET

Evaluating Complex Versus Defined Media

During the Lab

1 In the provided table, score the relative amount of growth in each tube. Compare the five tubes of each medium with each other, as well as the amount of growth for each organism in the various media. Use "0" for no growth, "3" for abundant growth, and "1" and "2" for degrees of growth in between. Use the most turbid of the 15 broths as your "3."

Specimen	Nutrient Broth	Brain-Heart Infusion Broth	Glucose Salts Broth	Interpretation (relative fastidiousness)
Uninoculated control				Not applicable

2 In the provided table, identify the carbon and nitrogen source(s) in each medium and then characterize each as "defined" or "complex." If the media is complex, list the ingredient(s) that make it so. Finally, use your results to characterize each medium based on its ability to support growth of a wide range of organisms (poor, fair, good).

Media Description	Nutrient Broth	Brain-Heart Infusion Broth	Glucose Salts Broth
Carbon source(s)			
Is this media complex or defined?			
Ingredient(s) making it "complex" (if applicable)			
Ability to support a wide range of organisms (poor, fair, good)			

Lab Related Questions

1 Which of the following is (are) classified as complex media? (Select all that apply.)

a. Glucose minimal salts broth
b. Brain-heart infusion broth
c. Blood agar
d. Nutrient broth
e. Agar

2 Select the true statement(s) about clinically important microbes (select all that apply).

a. Most can use solar energy.
b. Most require an organic carbon source.
c. Most are autotrophs.
d. Most are heterotrophs.
e. Most are chemotrophs.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 17
DATA SHEET
(continued)

3 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ Defined media are commonly used for isolating an unknown pathogen from a clinical sample.

_____ Complex media are precisely defined and replicable from batch to batch.

_____ Most bacteria are non-cultivable using current laboratory media.

_____ Adding agar to a medium makes it a complex medium.

4 In this exercise, we compared the _____ of each culture to determine how each media supported (or failed to support) the growth of a given microbe.

5 Select the false statement(s) about fastidious microbes. (You may select more than one answer choice.)

a. They are more likely to grow on defined media as compared to complex media.
b. They are more likely to be heterotrophs than autotrophs.
c. They include most clinically important microbes.
d. They can make all their own complex organic compounds from scratch.
e. They do not require a carbon source.

6 Select the ingredient(s) that would not be included in defined media. (You may select more than one answer choice.)

a. Yeast extract
b. Glucose
c. Water
d. Blood
e. Salt
f. Peptone

Critical Thinking

1 Use the provided data to fill in the missing data—blanks **a** through **f**—with the correct term, *growth* or *no growth*.

	Bacterium 1	Bacterium 2
Glucose Salts Broth	No growth	Growth
Glucose Salts Broth + Tryptophan (an amino acid)	No growth	a.
Glucose Salts Broth + Histidine (an amino acid)	b.	c.
Glucose Salts Broth + Peptone	No growth	d.
Glucose Salts Broth + Peptone + Sheep Blood	e.	f.
Sheep Blood Agar	Growth	Growth

2 Explain your reasoning for the response you entered in space "a" in the table.

3 Explain your reasoning for the response you entered in space "b" in the table.

4 Based on the data in the table in Question 1, would you classify Bacterium 1 as fastidious or non-fastidious? Explain your reasoning.

EXERCISE 18

Thioglycollate Broth

After doing this exercise, you should be able to:

1. Define aerotolerance and differentiate aerotolerance by culturing microbes in thioglycollate medium.
2. Describe how thioglycollate media allows us to determine a microbe's aerotolerance.
3. State the clinical importance of determining aerotolerance for infectious agents.
4. Apply aseptic bacterial transfer and culture techniques.

Background

Thioglycollate broth is an enriched (or complex) medium that usually contains yeast extract and pancreatic digest of casein. We use this generalized enriched recipe for this lab. However, if we were trying to grow especially fastidious bacteria, we would add a variety of supplements like hemin (an iron-containing compound extracted from red blood cells) and vitamin K to support their growth; recall that many pathogens are particularly fastidious.

A Note on Microbial Oxygen Tolerance

Most microorganisms survive within a range of environmental conditions, but not surprisingly, growth is optimal when conditions are ideal. Oxygen (O_2) levels especially influence microbial growth. **Aerotolerance** is the ability or inability to live in the presence of oxygen.

Some organisms, called **obligate aerobes**, require oxygen for their metabolic needs. These organisms, like us, require oxygen to extract energy from nutrients (to carry out aerobic respiration). On the opposite end of the aerotolerance spectrum, we encounter **obligate (strict) anaerobes**, which don't use oxygen gas for their metabolic pathways. Furthermore, obligate anaerobes don't survive for long in the presence of oxygen gas (O_2) because they can't sufficiently protect themselves from toxic oxygen intermediates—especially superoxide (O^{-2}) and hydrogen peroxide (H_2O_2)—that arise when oxygen participates in certain chemical reactions. Some microbes called **facultative anaerobes** grow in the presence *or* absence of oxygen. When oxygen is available, they use it to carry out aerobic respiration; but if oxygen is unavailable they either carry out fermentation or respire anaerobically (reducing sulfur or nitrate instead of oxygen). (Refer to Appendix A and Section 5 for more information on anaerobic respiration and fermentation.) Still other organisms, called **aerotolerant anaerobes**, are indifferent to oxygen; they can survive when it is around, but they don't use it. Lastly, **microaerophiles**, as the name suggests, survive only in environments containing lower than atmospheric oxygen levels. Specific microaerophiles, called **capnophiles**, only survive if O_2 levels are reduced and carbon dioxide (CO_2) levels are elevated. Of course, bacteria, to include those that are pathogens, are diverse in their oxygen needs and span the range of these described categories.

How Thioglycollate Media Reveals an Organism's Aerotolerance

As a complex medium, thioglycollate broth nutritionally supports the growth of a broad variety of organisms, but it is particularly well adapted for cultivating organisms that can't grow in atmospheric levels of oxygen. Why is that? The answer has two parts: (1) the effects of autoclaving and (2) some specialized ingredients. Let's tackle autoclaving effects first.

As with most media, before inoculation, we autoclave the thioglycollate media to sterilize it. This ensures we are specifically studying our target sample, but importantly this process also removes most of the medium's free oxygen gas (O_2). After autoclaving, the media cools and oxygen diffuses back into the tube media in a gradient manner, leveling out at about 20–21% O_2 at the top of the tube, about 2–10% O_2 toward the middle of the tube, and no oxygen gas in the bottom portion of the tube. Autoclaving alone doesn't allow us to establish an area of the tube that is verifiably free of oxygen gas. To maintain a verifiable absence of oxygen at the bottom of the tube, specialized ingredients and oxygen indicators are added to the medium.

The sodium thioglycollate (an organic sodium salt) and L-cystine (an amino acid) in the media reduce oxygen to water, which generates an anaerobic (oxygen-free) environment deeper in the tube. An oxygen detector called **resazurin** indicates if an oxygen-free environment exists at the bottom of the tube. Resazurin appears pink when oxidized, which occurs when oxygen gas is present, and colorless when reduced—when oxygen gas is absent (Fig. 18.1). A small amount of agar is also added to the medium; while it's not enough agar to solidify the media, it is enough to slow oxygen diffusion through the media.

Based on the generated oxygen gradient in thioglycollate media, we expect the following growth patterns that reveal an organism's aerotolerance (Fig. 18.2):

- **Obligate aerobes:** These organisms require oxygen to conduct aerobic respiration and therefore only thrive at the top of the medium.
- **Facultative anaerobes:** These microbes will grow throughout the medium, but will be denser near the top of the medium because of the higher ATP yield from aerobic respiration compared to fermentation or anaerobic respiration.
- **Aerotolerant anaerobes:** Indifferent to oxygen levels, these microbes live uniformly throughout the medium.
- **Microaerophiles:** These microbes grow in the middle region of the medium.
- **Obligate (strict) anaerobes:** Because even small amounts of oxygen are lethal to these organisms, they only grow toward the bottom of the medium.

FIGURE **18.1** **Aerobic and Anaerobic Zones in Thioglycollate Broth** • The pink region is the upper, aerobic portion of the broth results from oxidation of the indicator resazurin. In the lower, anaerobic portion of the tube, the dye is not reduced so the typical straw color of the medium shows.

FIGURE **18.2** **Growth Patterns in Thioglycollate Broth** • Compare these tubes to the uninoculated tube in Figure 18.1.

Clinical Applications

Thioglycollate broth is an enriched liquid medium that promotes growth of a wide variety of fastidious and nonfastidious microorganisms. While it can be used to grow and temporarily store and/or transport a pure culture, it is more often used to study an uncharacterized sample, which may or may not contain a mixture of bacteria with diverse aerotolerances. It's especially useful in diagnostic clinical microbiology because many pathogens are not only fastidious about their nutrients, but they're often particular about how much oxygen gas they encounter. Consequently, this media is primarily used to grow anaerobic and microaerophilic bacteria from blood samples. Thioglycollate media is also useful for isolating important pathogens such as *Clostridium* species. For example, *C. perfringens*, which causes gas gangrene, and *C. difficile*, which causes an infectious diarrhea that can progress to pseudomembranous colitis, are both obligate anaerobes.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Tubes of thioglycollate broth (4)
- ❑ Fresh broth cultures of these recommended bacteria:
  - ☐ *Alcaligenes faecalis* (subspecies *faecalis*) (recommend a BSL-1 strain ATCC 15554)
  - ☐ *Clostridium butyricum* (BSL-1 strain ATCC 19398)
  - ☐ *Staphylococcus epidermidis*

Medium Recipe

Thioglycollate Broth

Yeast extract	5.0 g
Pancreatic digest of casein	15.0 g
Dextrose (glucose) ($C_6H_{12}O_6$)	5.5 g
Sodium chloride (NaCl)	2.5 g
Sodium thioglycollate	0.5 g
L-cystine ($C_6H_8O_4(NH_2)_2S_2$)	0.5 g
Agar	0.75 g
Resazurin	0.001 g
Distilled or deionized water	1.0 L

PROCEDURE

Today you will be inoculating three different bacteria into thioglycollate broth to determine their aerotolerance. Your instructor may have additional organisms or may have you collect environmental samples to test. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

1. **Obtain Your Media:** Working with your group, obtain four tubes of thioglycollate media and label them with your name, the date, and the medium's name.
2. **Label Your Tubes:** Label three of the tubes with the name of an organism (add a different organism name on each tube) and label the fourth tube "Uninoculated."
3. **Inoculate Your Media with the Designated Bacteria:** Using your loop, inoculate three broths with the organisms provided. (*Note:* When inoculating thioglycollate broth, it helps to dip the loop all the way to the bottom of the tube and gently mix the broth with the loop as you remove it. Do not shake the tube. Finish mixing by gently rolling the tube in an upright position between your hands.) Do not inoculate the fourth tube.
4. **Incubate the Samples:** Incubate all tubes (including the uninoculated ones) at 35 ± 2°C for 24–48 hours.
5. **Clean up:** Save or dispose of the original cultures as your instructor directs.

→ Next Lab Session

1. **Obtain Your Samples:** Collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Observe the Samples:** Using the uninoculated control as a comparison, examine and note the location of the growth in all tubes.
3. **Record your results:** Note all observations and results on the data sheet at the end of this lab. (*Note:* Results may vary. Record what you see, not what you expect or what other groups are getting for results.) Also, note any changes that may have occurred as a result of incubation, especially in the colored region at the surface.
4. **Clean up:** Dispose of your samples as your instructor directs.

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 18
DATA SHEET

Thioglycollate Broth

During the Lab

1 In the table provided, list three pieces of information for each of your four tubes:

- First, sketch each culture showing the location of growth.
- Second, indicate the amount of growth in the aerobic and anaerobic regions using the following system:
  0 = no growth
  1–2 = moderate growth
  3 = abundant growth
- Third, place each organism into an aerotolerance category.

Specimen	Location of Growth in Medium	Aerotolerance Category
Uninoculated control		Not applicable

2 Additional observation notes (e.g., media coloration).

Lab Related Questions

1 Why is it essential that you avoid mixing/agitating thioglycollate broth at all points in this lab exercise? (Select all that apply.)

a. Mixing can disrupt the oxygen gradient in the media.
b. Mixing can change the nutrient gradient in the broth.
c. Mixing increases the risk of contaminating the sample.
d. Mixing can disperse the bacteria into areas where they did not actually grow.
e. Mixing can alter the aerotolerance of the samples inoculated into the media.

2 Select the true statement(s) about thioglycollate broth (select all that apply).

a. The dye resazurin removes oxygen from the media.
b. This media is useful for growing fastidious microbes.
c. This media can grow organisms that may not tolerate atmospheric oxygen levels.
d. When fresh, this media is normally pink throughout.
e. Thioglycollate broth is used to detect the presence of pathogens in clinical blood samples.

3 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ If growth is absent at the top of the tube, it means obligate aerobes did not grow.

_____ Agar is added to thioglycollate medium to solidify it.

_____ If growth is absent at the bottom of the tube, it means obligate anaerobes did not grow.

_____ Regarding nutrients, this medium is considered a defined media.

4 Match the terms to their descriptions. Descriptions can be used more than once, and the terms may have more than one appropriate description, in which case you should include all the relevant descriptions from the list.

Terms:

_____ Obligate anaerobe

_____ Microaerophile

_____ Facultative anaerobe

_____ Obligate aerobe

_____ Capnophile

_____ Aerotolerant anaerobe

Descriptions:

a. Cannot grow in the presence of oxygen gas.
b. Grow in the absence of oxygen.
c. Cannot grow without gaseous oxygen.
d. Require increased CO_2 levels to grow.
e. Require reduced gaseous oxygen levels to grow.
f. Grow in the presence of oxygen.
g. Include pathogens.
h. Use oxygen in their metabolic processes.
i. Do not use oxygen in their metabolic processes.

5 Old thioglycollate can give inaccurate data. What could you expect if older thioglycollate medium was used? (Select all that apply.)

a. Fastidious bacteria would be unable to grow.
b. Strict anaerobes may not be detected.
c. Obligate aerobes would grow less than expected.
d. Microaerophiles would grow less than expected.
e. Aerotolerant anaerobes would be unable to grow.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 18
DATA SHEET
(continued)

6 Thioglycollate media has an oxygen gradient and maintains it for the medium's normal shelf life due to (select all that apply):

a. The additive sodium thioglycollate.

b. The additive CO_2.

c. Resazurin.

d. L-cystine.

e. Autoclaving effects.

f. The presence of a high agar concentration.

Critical Thinking

1 Thioglycollate medium is useful for characterizing the microorganisms present in blood cultures. However, interpreting blood cultures is challenging since normal microbiota (normal flora) from the skin at the venous blood draw site can contaminate the samples. For this reason, blood is usually taken from each arm and the growth from the blood draw of the right arm is compared to that of the sample from the left arm. Imagine you obtain the following blood sample data:

Sampled Site	Growth Detected	Growth Location in Thioglycollate Medium
Right arm	Yes	Abundant bacterial growth at the top of the tube and bacterial growth isolated at the bottom of the tube
Left arm	Yes	Bacterial growth isolated at the bottom of the tube; no other growth observed

Answer the following based on the data:

a. Would you conclude normal microbiota were detected? Explain your reasoning.

b. Would you conclude the patient has an anaerobic blood infection (bacteremia)? Explain your reasoning.

2 Oxygen is usually abundant in the lungs, so why do some bacteria that cause lung infections (e.g., *Mycobacterium tuberculosis*, the bacterium that causes tuberculosis) evolve to be facultative anaerobes?

EXERCISE 19

Anaerobic Jar

After doing this exercise, you should be able to:

1. State why anaerobic jars are useful in the microbiology lab setting.
2. Describe the different versions of anaerobic jars and how they work.
3. State the clinical usefulness of anaerobic jars.
4. Apply aseptic bacterial transfer and culture technique to grow bacteria under anaerobic conditions in an anaerobic jar.

Background

In Exercise 18, we explored thioglycollate broth and the specifics regarding bacterial classifications by aerotolerance (obligate aerobes, facultative anaerobes, aerotolerant anaerobes, microaerophiles, and strict anaerobes). We also learned that thioglycollate is an enriched liquid medium that allows us to culture fastidious bacteria that may require reduced oxygen levels, or even anaerobic conditions to grow. And while thioglycollate broth is useful for isolating an unknown bacterium present in a specimen (e.g., patient blood samples) and/or maintaining certain samples for shorter term storage and transport, the oxygen gradient dissipates over time as oxygen diffuses through the media. Another limitation of thioglycollate broth is that, aside from revealing what oxygen levels an organism can tolerate, it does not reveal much else about the microbe's metabolic features. In such a case, we'd need to grow our bacterium on specialized biochemical test media while simultaneously maintaining the culture under the bacterium's optimal atmospheric growth conditions. An aerobic jar facilitates this.

Various airtight jars and pouches allow us to culture obligately anaerobic, microaerophilic, or capnophilic microorganisms under their preferred oxygen conditions without using an anaerobic incubator and/or a CO_2 incubator. Anaerobic jars are also an ideal way to store and transport anaerobic cultures without having to first transfer the cultures to thioglycollate media. Anaerobic jar setups vary in size, but typically accommodate multiple agar plates and/or test tubes and still fit in a standard incubator. The GasPak 100 Anaerobic Jar by Becton Dickinson (BD) is an example (Fig. 19.1), it holds up to 12 petri plates.

Each system can be used with a variety of gas generating sachets (packets) that produce the desired atmosphere. For instance, BD GasPak EZ Anaerobe Sachets contain water, ascorbic acid, activated carbon, and inorganic carbonate. When removed from their wrapper and sealed in the jar, the contents are exposed to air and ascorbic acid reacts with O_2 to form dehydroascorbic acid and water (Fig. 19.2), thus making the interior of the jar anaerobic (<1% O_2). The inorganic carbonate reacts to produce an atmosphere with roughly 13% carbon dioxide (air is 0.04% CO_2).

With a slightly different composition, the BD GasPak EZ Campy Sachets ("Campy" stands for *Campylobacter jejuni*, a microaerophilic pathogen) produce microaerophilic conditions (6–16% O_2). The inorganic carbonate produces an atmosphere of 2–10% CO_2.

FIGURE 19.1 An Anaerobic Jar • The large white packet inserted between the jar and the plates performs the chemistry required to produce anaerobic conditions within the jar. Condensation within the jar is an indication that the packet has performed its chemistry, but doesn't verify that anaerobic conditions have been produced. The white strip at the top contains a dye that is colorless when oxygen is absent.

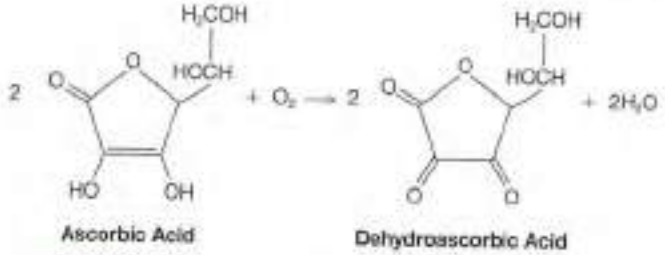


FIGURE 19.2 Reaction of Ascorbic Acid and Oxygen • When exposed to air in the sealed anaerobic jar, ascorbic acid reacts with oxygen to produce dehydroascorbic acid and water to make the atmosphere within the jar anaerobic. This water is often visible as condensation inside an anaerobic jar.

Oxoid AnaeroGen Atmospheric Generation Systems are an alternative to the BD systems and work in a similar way. The advantages of both systems over previous ones are that they don't require addition of water or a palladium catalyst; and, most importantly, they don't produce flammable H_2 gas as a byproduct. An indicator strip is used with the anaerobic systems to ensure that anaerobic conditions have been created. The indicator strip is soaked with methylene blue or resazurin dye that appears blue or pink, respectively, when oxidized and colorless when reduced.

Clinical Applications

Anaerobic jars are a convenient and relatively inexpensive way to grow and study anaerobic and/or microaerophilic bacteria in a standard incubator. These jars typically accommodate plated and/or broth media. This expands the types of media we can use to grow these oxygen-intolerant bacteria beyond the isolation media, thioglycollate broth (see Ex. 18). Growing microbes on diverse biochemical media is often important for isolating and better characterizing infectious agents. Also, for example, if we wished to perform an antibiotic sensitivity test on an anaerobic bacterium that was causing an infection, we'd need to incubate the sample plates under anaerobic conditions. Anaerobic jars also make it easier to transport anaerobic specimens to another laboratory, if needed.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Anaerobic jar with anaerobic gas generator packet (1)
- ❑ Nutrient agar (NA) plates (2)
- ❑ Fresh broth cultures of these recommended bacteria:
  - ☐ *Alcaligenes faecalis* (subspecies *faecalis*) (recommend a BSL-1 strain ATCC 15554)
  - ☐ *Clostridium butyricum* (BSL-1 strain ATCC 19398)
  - ☐ *Staphylococcus epidermidis*

PROCEDURE

Today you will be inoculating different bacteria onto two nutrient agar plates. One plate will be incubated in an anaerobic jar and the other will be grown under standard conditions. This will allow you to make a comparison regarding which microbes grew under anaerobic versus aerobic conditions. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

1. **Obtain Your Media:** Working with your group, obtain two nutrient agar plates.
2. **Label Your Tubes:** Using a marking pen, divide the bottom of each plate into three sectors (or more, if your instructor has additional organisms for you to use). Label each plate with your name, the date, and organism by sector.
3. **Inoculate Your Media with the Designated Bacteria:** Using your loop, inoculate corresponding sectors of both plates with the organisms provided. (*Note:* Inoculate with single streaks about 1 cm long.) Tape the lids in place.
4. **Place Your Anaerobic Plate in the Anaerobic Jar:** Place one of your plates into the anaerobic jar in an inverted position. Depending on your class size, more than one jar may be necessary. When all groups have placed their plates in the jar, discharge the packet as follows.

- If using a BD system, follow the packet instructions, but they should be something like this:
  - a Stick the methylene blue strip on the wall of the jar.
  - b Open the packet and place it in the jar with the label facing inward.
  - c Immediately close the jar.
  - d Examine for condensation within about 30 minutes. If none appears, start over with a new packet.
- If using an Oxoid system, follow the packet instructions, but they should be something like this:
  - a Stick the resazurin indicator strip on the wall of the jar.
  - b Open the foil packet and remove the sachet. Place the sachet in the jar.
  - c Close the jar within 1 minute.
  - d Examine for condensation within about 30 minutes. If none appears, start over with a new packet.

Once the anaerobic packet is deemed to be working, the sealed anaerobic jar is placed into a standard incubator set to 35 ± 2°C. The samples will be incubated for 24 to 48 hours.

5. **Place Your Aerobic Plate in the Incubator:** Place your second plate (the aerobic plate) in a standard incubator set to 35 ± 2°C. Incubate the samples for 24 to 48 hours.
6. **Clean up:** Save or dispose of the original cultures as your instructor directs.

Next Lab Session

1. **Obtain Your Samples:** Collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Observe the Samples:** Examine and compare the growth on the plates. (*Note:* Density of growth will be the most useful basis for comparison.)
3. **Record Your Results:** Note all observations and results on the data sheet at the end of this lab.
4. **Clean up:** Properly dispose of the packet in laboratory waste. The Oxoid packet may still be warm to the touch. Let it cool before disposing of it. Dispose of your samples as your instructor directs.

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 19

DATA SHEET

Anaerobic Jar

During the Lab

1 In the table provided, indicate the amount of growth of each organism on each plate using the following system:

0 = no growth

1–2 = moderate growth

3 = abundant growth

Organism	Growth on Aerobic Plate	Growth on Anaerobic Plate	Aerotolerance Category

Lab Related Questions

1 Suppose that after incubation you examine the jar before opening it and see that the methylene blue strip is blue (or the resazurin strip is pink). What is the *best* conclusion about the internal jar environment in such a case?

a. It became aerobic upon being exposed to room temperature.

b. It was anaerobic for most of the incubation period.

c. It was aerobic for most of the incubation period.

d. It was aerobic for at least a portion of the incubation period.

e. It was incubated at the wrong temperature.

2 How would you expect the growth on the plates inside the jar to differ from the plates incubated outside the jar? (Select all that apply.)

a. Obligate aerobes will grow best inside the jar apparatus.

b. Obligate anaerobes will grow best inside the jar apparatus.

c. Facultative anaerobes will grow best inside the jar apparatus.

d. Obligate aerobes will grow best outside the jar apparatus.

e. Facultative anaerobes will grow best outside the jar apparatus.

3 An alternative to the anaerobic jar is a candle jar, in which a candle is placed in the jar, lit, and the lid closed to enable the flame to use the available oxygen. Typically, in this system, not all of the oxygen is used. Which organisms would most likely benefit from this environment? (Select all that apply.)

a. Obligate anaerobes

b. Aerotolerant anaerobes

c. Obligate aerobes

d. Facultative anaerobes

e. Microaerophiles

4 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ Anaerobic jars are rendered anaerobic by a chemical reaction in which atmospheric oxygen is chemically converted to CO_2.

_____ Anaerobic jars will only permit the growth of obligate anaerobes.

_____ Aerotolerant anaerobes should grow in an anaerobic jar, but such a jar is not necessary for their cultivation.

_____ Anaerobic jars are only used with thioglycollate media.

5 Which of the following represent an advantage that anaerobic jars have over thioglycollate media?

a. Anaerobic jars are portable.
b. Anaerobic jars can allow for anaerobic incubation of diverse media formats.
c. Anaerobic jars are useful for growing anaerobes.
d. Anaerobic jars are useful for growing facultative anaerobes.
e. Anaerobic jars are useful for isolating species with an unknown aerotolerance.

6 Which of the following is (are) likely to lead to the anaerobic conditions in an anaerobic jar being disrupted? (Select all that apply.)

a. Opening the jar during incubation.
b. Improperly sealing the jar.
c. Using an expired chemical pack to deoxygenate the jar.
d. Incubating the jar outside of a 35 ± 2°C range.
e. Moving the jar.

7 The dye _____ or _____ is used to indicate that an anaerobic environment was achieved/maintained in an anaerobic jar. When oxygen is absent, these dyes appear _____.

Critical Thinking

1 Assume you are studying a newly discovered pathogen. You obtain the following data for two different isolates of the bacteria that were inoculated onto nutrient agar and then incubated at 35 ± 2°C under the conditions noted in the table.

Standard Incubator		Anaerobic Jar Incubation	
Observation 24-hours post-inoculation		Observation 24-hours post-inoculation	
Sample Isolate	Growth detected?	Sample Isolate	Growth detected?
1	Yes (abundant)	1	Yes (moderate)
2	Yes (absent)	2	Yes (abundant)
Observation 48-hours post-inoculation		Observation 48-hours post-inoculation	
Sample Isolate	Growth detected?	Sample Isolate	Growth detected?
1	Yes (abundant)	1	Yes (abundant)
2	Yes (absent)	2	Yes (abundant)

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 19
DATA SHEET
(continued)

Answer the following questions based on the data:

a. Based on these data, what can you conclude about Isolate 1? Support your answer by referring to specific data from the tables provided.

b. Based on these data, what can you conclude about Isolate 2? Support your answer by referring to specific data from the tables provided.

2 Considering the gaseous changes resulting from the burned candle, which group would benefit the most from a functional candle jar? (***Hint***: Think about a gas that is not involved in aerotolerance.)

— Lab Notes —

EXERCISE 20

The Effect of Temperature on Microbial Growth

After doing this exercise, you should be able to:

1. Explain why prokaryotes are especially sensitive to environmental pH and temperature shifts.
2. Define the terms *optimal temperature, minimum temperature, maximum temperature,* and *cardinal temperatures.*
3. Describe the temperature conditions that support the growth of psychrophiles, psychrotrophs, mesophiles, thermophiles, and extreme thermophiles.
4. Apply aseptic bacterial transfer and culture technique to set up an experiment that will assess the cardinal temperatures for a selection of bacteria.

Background

Even minor environmental changes can dramatically affect microbes, especially prokaryotes. Seemingly small shifts in environmental pH and/or temperature can be a life or death matter, because such factors dramatically change a prokaryote's ability to transport materials across the membrane, maintain membrane fluidity, perform complex enzymatic reactions, and maintain critical cytoplasmic pressure. This is in part why we are so detailed about incubation temperatures and the pH of the media we use in the laboratory setting.

One way to study how microbes respond to environmental changes is to alter an external factor and measure the resulting effect on the microbe's growth rate; that is, the cell density after a given incubation time. Such studies help us classify organisms by their preferred oxygen ranges (see Ex. 18), based on their preferred temperature growth range (this exercise), and according to their preferred pH growth range (covered in Ex. 21).

Prokaryotes and Temperature Tolerance

Prokaryotes have been discovered living in habitats ranging from −10°C to more than 110°C. Because microbes cannot regulate their internal temperature in response to their surroundings, they instead create proteins and membranes that function within a particular temperature range. As such, each species is characterized by a minimum, maximum, and optimum temperature, collectively known as its **cardinal temperatures** (Fig. 20.1).

Minimum and maximum temperatures are, simply, the temperatures below and above which the organism will not survive. Optimum temperature is the temperature at which an organism grows the fastest, its highest growth rate. The optimum temperature is usually presented as a tight range of temperatures within the minimum and maximum range; it is not usually defined as a single temperature point.

Classifying Prokaryotes by Temperature Growth Range

Prokaryotes that prefer to grow below 20°C are called **psychrophiles**. These are common in ocean, Arctic, and Antarctic habitats where the temperature remains permanently cold with little or no fluctuation. Organisms adapted to cold habitats that fluctuate from about 0°C to above 30°C are called **psychrotrophs.** Because psychrotrophic bacteria grow at room temperature as well as in refrigerated and frozen foods, they are associated with foodborne illness. Bacteria adapted to temperatures between 15°C and 45°C are known as **mesophiles.** Most bacterial residents in the human body (normal microbiota), as well as numerous human pathogens, are mesophiles. **Thermophiles** are organisms adapted to temperatures above 40°C. Thermophilic prokaryotes are found in composting organic material, soil surfaces subjected to direct sunlight, and silage. Prokaryotes isolated from ocean floor hydrothermal vents and other geothermal sites are called **extreme thermophiles** because they can survive temperatures in the 65°C and 110°C range (up to 122°C for *Methanopyrus*). Extreme thermophiles grow best above 80°C. Figure 20.2 illustrates typical temperature ranges and classifications for the prokaryotes (domains Bacteria and Archaea).

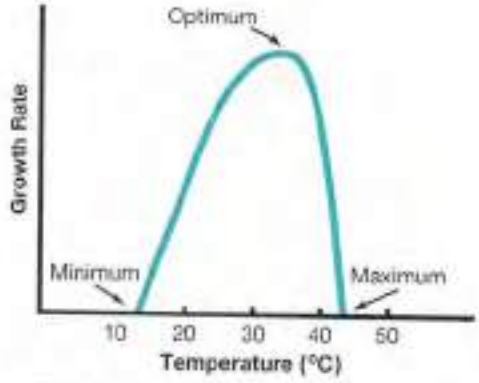


FIGURE **20.1** **Typical Temperature Growth Range for Mesophiles** • The "minimum" and "maximum" are temperatures beyond which no growth takes place. The "optimum" is the temperature at which growth rate is highest. These are the organism's "cardinal temperatures."

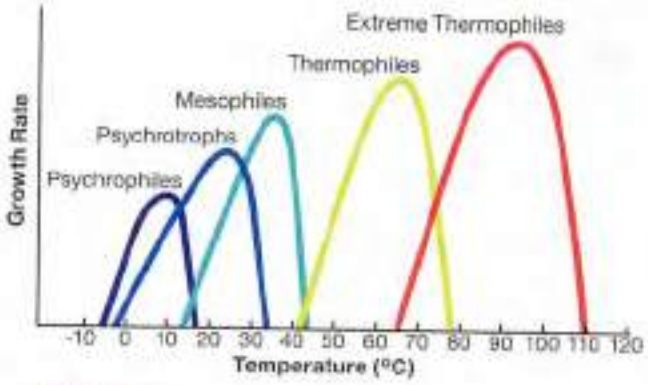


FIGURE **20.2** **Thermal Classifications of Bacteria** • This graph shows that bacteria can be grouped into five main categories based on the temperatures at which they can grow. The temperature data represented here are generalizations for each group.

Clinical Applications

This is a qualitative procedure that allows us to study how temperature affects microbial growth. It allows us to estimate the cardinal temperatures for individual species rather than for an entire group, as shown in Figure 20.2.

Understanding the cardinal ranges for temperature growth is certainly important for culturing bacteria in the laboratory, but it also has important pathophysiological consequences. Most pathogenic bacteria are mesophiles; however, even within these groupings, we find different optimal temperatures and even growth ranges for different bacterial species.

For example, *Mycobacterium leprae*, the pathogen that causes leprosy, is a mesophile with an optimal growth temperature of 33–35°C. Because this bacterium's optimal growth temperature is below the normal core human body temperature (which is 36.5–37.5 °C), *M. leprae* mainly infects the superficial peripheral nerves, the nasal passages, and the skin, areas where the bacterium's optimal growth temperature range is met. In contrast, we find the causative agent of tuberculosis (*Mycobacterium tuberculosis*) in the same genus as *M. leprae*. It is another example of a mesophile; however, it has an optimal growth temperature that aligns with normal core human body temperature. If *M. tuberculosis* could not grow at core human temperature, it would certainly struggle to infect deep in the lungs as it does.

Further complicating matters, we find that some bacteria have an exceedingly broad temperature growth range. For example, *Listeria monocytogenes*, which causes a foodborne infection called listeriosis, has a growth range that spans -1.5–45°C. This broad temperature tolerance means it can easily grow in refrigerated foods, but its optimal growth temperature spans our normal core body temperature, making it possible for the bacterium to cause gastrointestinal infections.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Sterile tryptic soy broths (TSB) (or nutrient broths, NB) (25 total tubes)
- ❑ Tryptic soy agar (TSA) plates (2)
- ❑ Sterile transfer pipettes (4)
- ❑ Fresh broth cultures of these recommended bacteria:
  - ☐ *Escherichia coli*
  - ☐ *Geobacillus (Bacillus) stearothermophilus*
  - ☐ *Serratia marcescens*
  - ☐ *Pseudomonas fluorescens*
- ❑ Incubating devices set at 10°C, 25°C, 35°C, 45°C, and 60°C (5 total). These five devices may be any combination of the following:
  - ☐ refrigerator
  - ☐ incubator
  - ☐ hot-water bath
  - ☐ cold-water bath

PROCEDURE

Today you will examine the growth characteristics of four organisms at five different temperatures. You'll also observe how temperature influences pigment production in *Serratia marcescens*. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

1 **Obtain Your Media:** Working with your group, obtain 25 tryptic soy broths (TSB) and two TSA plates.

2 **Label Your Tubes:** Label your tubes so that you have designated one broth for each organism at each temperature plus one for an uninoculated control (Fig. 20.3). Label one TSA plate for 20°C *S. marcescens* and the other for 35°C *S. marcescens*. Each student should choose one or two stock cultures from the options your instructor provides to inoculate. Divide the work load with your lab partner(s) as evenly as possible.

3 **Inoculate Your Media with the Designated Bacteria:**

- **Plate Inoculations:** To inoculate each TSA plate with *Serratia marcescens*, use an inoculation loop and perform the quadrant streak plate isolation method as described in Exercise 4.
- **Broth Inoculations:** Mix each stock sample thoroughly, but be careful not to splash the culture into the tube cap. Then, using a sterile transfer pipette, inoculate each set of TSB tubes with 1 drop of organism (be consistent). Refer to Figure 20.3 for guidance on this. You may fill the pipette with about 1 mL of broth and deliver the drops with the same pipette to the tubes without flaming them. In your set of tubes, leave one tube uninoculated.

CAUTION!

- Before delivering the inoculum, insert the pipette well into the tube (but do not touch the broth).
- Do not spatter as you deliver the drop to each tube.
- Do not accidentally drip organism between tubes. If you do, then clean up the spill with proper decontamination approaches as your instructor directs.
- If you don't have enough organism left in the pipette to inoculate a tube, get more organism. You must not empty ("blow out") the pipette because you will create aerosols.
- Once finished, properly dispose of the pipette.

4 **Incubate the Samples:** Incubate all the TSB tubes at their appropriate temperatures for 24 to 48 hours. Place one of your TSB plates in a 20°C incubator and the other in a 35°C incubator and incubate the samples for 24 to 48 hours. Remember to invert your plates for incubation so condensation won't drip onto the agar surface.

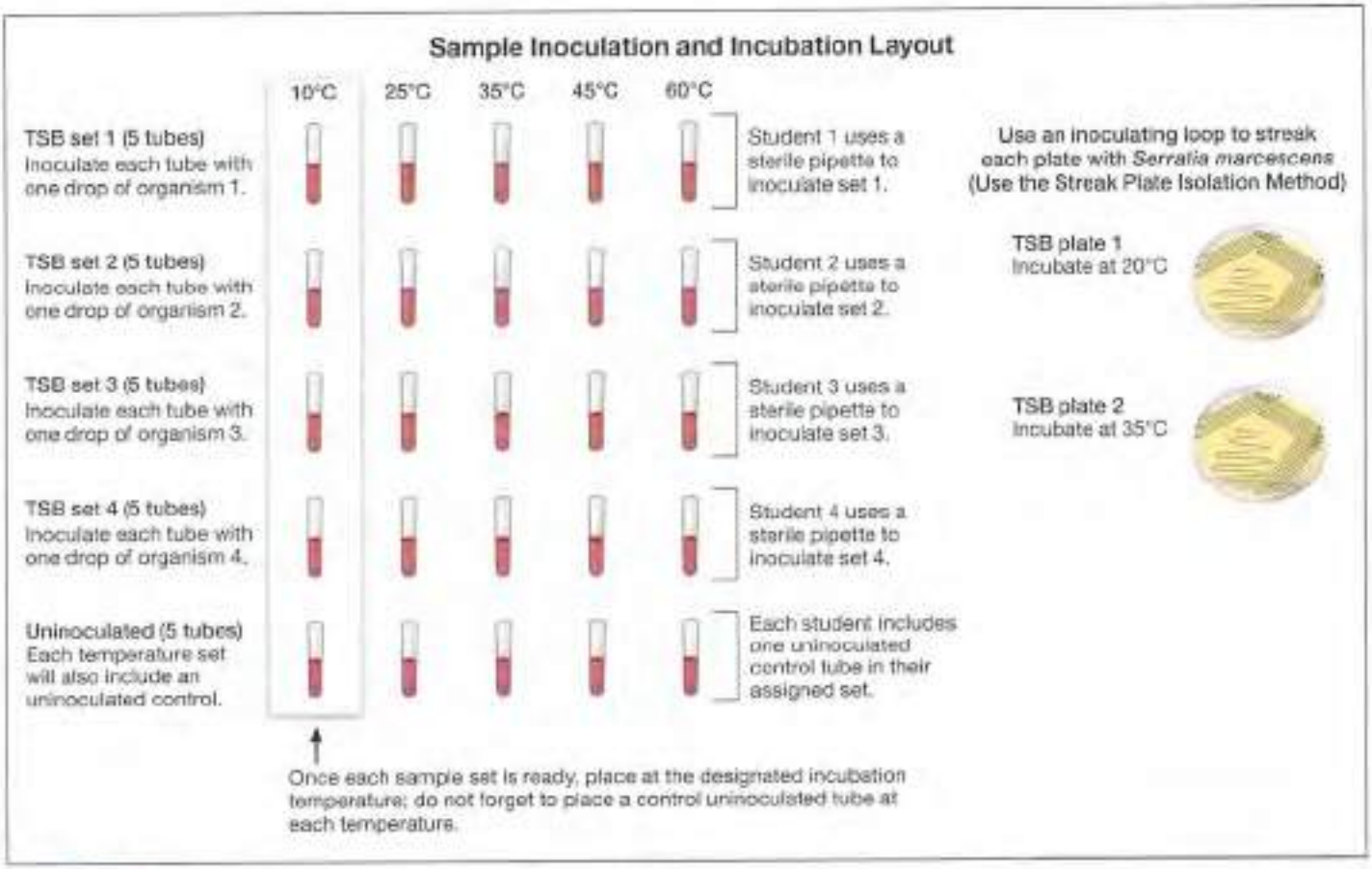

Sample Inoculation and Incubation Layout
10°C
25°C
35°C
45°C
60°C
TSB set 1 (5 tubes)
Inoculate each tube with one drop of organism 1.
Student 1 uses a sterile pipette to inoculate set 1.
TSB set 2 (5 tubes)
Inoculate each tube with one drop of organism 2.
Student 2 uses a sterile pipette to inoculate set 2.
TSB set 3 (5 tubes)
Inoculate each tube with one drop of organism 3.
Student 3 uses a sterile pipette to inoculate set 3.
TSB set 4 (5 tubes)
Inoculate each tube with one drop of organism 4.
Student 4 uses a sterile pipette to inoculate set 4.
Uninoculated (5 tubes)
Each temperature set will also include an uninoculated control.
Each student includes one uninoculated control tube in their assigned set.
Once each sample set is ready, place at the designated incubation temperature; do not forget to place a control uninoculated tube at each temperature.
Use an inoculating loop to streak each plate with Serratia marcescens (Use the Streak Plate Isolation Method)
TSB plate 1
Incubate at 20°C
TSB plate 2
Incubate at 35°C

FIGURE **20.3** Broth and Plate Sample Organization and Inoculation Process ■

5 **Clean up:** Save or dispose of the original cultures as your instructor directs.

→ Next Lab Session

1 **Obtain Your Samples:** Collect your cultures from the incubator (or wherever your instructor placed your samples).

2 **Clean the Outside of Each Broth Tube:** Use a clean tissue to wipe the glass of each broth tube and place each tube in a test-tube rack organized into groups by organism.

3 **Observe Your Broth Sample Data and Record the Results:** Gently mix each tube until you obtain uniform turbidity (cloudiness). Be careful not to splash the culture into the caps. Compare all tubes of a species to each other. Rate each as 0, 1, 2, or 3, according to its turbidity (0 is clear—the uninoculated tube—and 3 is the most turbid.) Note your observations and results on the data sheet at the end of this lab.

4 **Analyze Your Broth Sample Data:** Use the data you recorded in Step 3 to make a graph. Use the graph paper provided with the data sheet at the end of this exercise. On your graph, plot the relative turbidity values versus temperature for each organism. If you need help with graphing, see pages 10–12.

5 **Develop Conclusions Based on the Broth Growth Data:** On your graph, indicate the optimum temperature for each bacterium studied. Then use brackets to indicate the temperature range for each. (*Note:* This also indicates the minimum and maximum temperatures.) Finally, use this data to develop a conclusion about the temperature classification for each organism.

6 **Observe the Plate Samples and Record the Results:** Now, examine the *Serratia marcescens* plates that were incubated at two different temperatures. Compare the growth characteristics for each plate and enter your observations and results on the data sheet at the end of this lab.

7 **Clean up:** Properly dispose of your samples as your instructor directs.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 20

DATA SHEET

The Effect of Temperature on Microbial Growth

During the Lab

1 For each organism, in the table provided record the turbidity (cloudiness) of the sample. The cloudier the sample, the more growth. Use your uninoculated control as a basis for your "0" point.

0 = no growth (completely clear sample)

1–2 = moderate growth

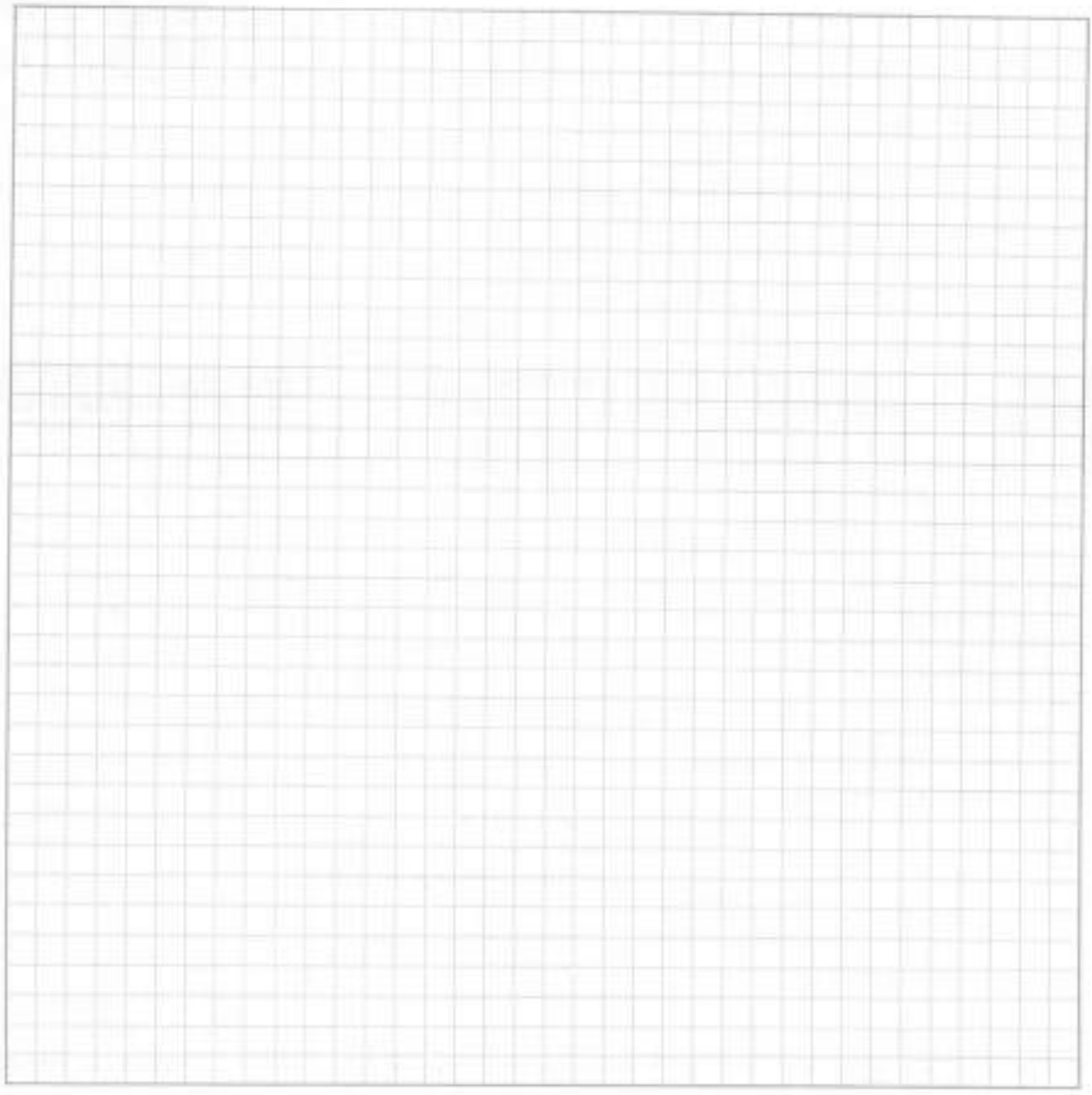
3 = abundant growth

Broth Data						
Organism	10°C	25°C	35°C	45°C	60°C	Temperature Range Classification

2 In the table provided, record the growth characteristics of the *Serratia marcescens* incubated at two temperatures.

Plate Data	
Incubation Temperature	Description of Growth
20°C	
35°C	

3 Plot relative turbidity (y-axis numeric values will be 0, 1, 2, 3) versus temperature (x-axis values will be the five tested temperatures) on the graph paper provided. If you need help with graphing, see pages 10–12.



4 Using the data in your graph, determine the cardinal temperatures for each of the four organisms. Indicate the optimum temperature for each organism on your graph and use brackets to designate the range (minimum to maximum) for each.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 20
DATA SHEET
(continued)

Lab Related Questions

1 From the following, what is the *best* conclusion about mesophiles?

a. They do not grow at room temperature.
b. They do not cause disease.
c. They are pathogens.
d. They would grow at 0°C.
e. They would not grow at 70°C.

2 In this experiment, _____ is the independent variable.

3 In this experiment, _____ is the dependent variable.

4 The organisms most likely to cause frozen food spoilage are (select all that apply):

a. Pathogens.
b. Psychrophiles.
c. Psychrotrophs.
d. Mesophiles.
e. Prokaryotes.

5 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ Mesophiles include pathogens.

_____ The optimal temperature is the warmest temperature that supports a given microbe's growth.

_____ An organism's optimum temperature is usually a tight range of temperatures within the minimum and maximum range.

_____ All mesophiles have the same optimal growth temperature.

6 Match the terms to their most appropriate descriptor.

_____ Thermophiles	a. Grow best below 0°C.
_____ Psychrophiles	b. Grow best above 80°C.
_____ Mesophiles	c. Include most human pathogens.
_____ Extreme thermophiles	d. Are likely to live in composting organic material.
_____ Psychrotrophs	e. Are adapted to habitats ranging from about 0°C to above 30°C.

7 Choose the true statement(s) about mesophiles (select all that apply).

a. They are all able to cause disease.
b. They are characterized by the same optimal growth temperature.
c. They are characterized by the same minimum and maximum growth range.
d. They include normal microbiota.
e. None of the above are true.

Critical Thinking

1 A classic Thanksgiving meal often includes a stuffed turkey. However, for food safety reasons, the recommendation is to cook the turkey separately from the stuffing. In the context of this lab, why does this advice make sense?

2 Different animals have different normal body temperatures. For example, chickens have a normal body temperature of 40.6–41.7°C (105–107°F). How might different animal body temperatures affect their susceptibility to certain bacterial pathogens?

EXERCISE 21

The Effect of pH on Microbial Growth

After doing this exercise, you should be able to:

1. Explain why prokaryotes are especially sensitive to environmental pH shifts.
2. Define the terms *optimal pH*, *minimum pH*, *maximum pH*, and *cardinal pH range*.
3. Describe the pH conditions that support bacterial growth.
4. Apply aseptic bacterial transfer and culture technique to set up an experiment that will assess how pH variation affects bacterial growth.

Background

Solution pH is an important chemical property, it reflects the concentration (or activity) of hydrogen (H^+) ions in a solution. We report pH using a logarithmic scale that ranges from 0–14. A pH of 7 is neutral, a pH less than 7 is acidic, and a pH above 7 is basic.

We can calculate pH using the following formula:

$$pH = -\log [H^+]$$

For example, let's assume we have an aqueous (water-based) solution that contains 10^{-6} moles of dissociated hydrogen ions per liter (mol/L). In this case, the concentration of H^+ (represented as $[H^+]$ in the formula) is 10^{-6} mol/L. Knowing this, we can determine the solution's pH as follows:

$$pH = -\log [H^+]$$

$$pH = -\log (10^{-6})$$

$$pH = -(-6)$$

$$pH = 6$$

Pure water contains 10^{-7} moles of hydrogen ions per liter and therefore has a pH of 7 and is neutral. As hydrogen ions increase, the pH decreases. As H^+ concentrations decrease, the solution pH increases (Fig. 21.1).

Solution Classification Acidity/Alkalinity	pH	H^+ Concentration in Moles/Liter	Common Examples	Organismal Classification
↑ Acidic	0	10^{0}	Nitric acid	↑ Acidophiles
	1	10^{-1}	Stomach acid	
	2	10^{-2}	Lemon juice	
	3	10^{-3}	Vinegar, cola	
	4	10^{-4}	Tomatoes, orange juice	
	5	10^{-5}	Black coffee	
	6	10^{-6}	Urine	
Neutral	7	10^{-7}	Pure water	Neutralophiles
Alkaline ↓	8	10^{-8}	Seawater	
	9	10^{-9}	Baking soda	Alkaliphiles ↓
	10	10^{-10}	Soap, milk of magnesia	
	11	10^{-11}	Ammonia	
	12	10^{-12}	Lime water [$Ca(OH)_2$]	
	13	10^{-13}	Household bleach	
	14	10^{-14}	Drain cleaner	

FIGURE **21.1** The pH Scale •

Prokaryotes and pH Tolerance

Even small changes in pH can have dramatic biochemical consequences. We see this in human beings; even minor blood pH shifts outside of the normal 7.35–7.45 pH triggers lightheadedness and fainting (syncope), and larger shifts can be deadly. Of course, prokaryotes are also sensitive to pH shifts. Changes to pH outside the range to which they are adapted may damage cellular protein structure (a process called denaturation) and destroy necessary membrane potential (used in ATP production). You can easily see protein denaturation in response to pH changes by adding lemon juice to milk. The curdling you would witness in this scenario is a protein denaturation event; casein, a protein in milk, denatures below pH 4.6. Just as we can't uncurdle milk (we can't restore the casein to its prior form), cells are often unable to restore a normal protein structure to their proteins once they are denatured. Consequently, these adverse effects may be irreversible and therefore lethal if widespread in a cell.

Most cells, including bacteria, use substances called buffers to maintain a near-neutral intracellular environment. Without buffers, the acids that cells make as a byproduct of their metabolic activities (e.g., acids made in carbohydrate fermentation or basic byproducts generated in protein metabolism) could shift intracellular pH enough to damage cellular proteins. Just as it is essential to maintain pH within a cell, when we are growing bacteria, it can be helpful to maintain an optimal growth pH. For this reason, we sometimes add buffers to bacteriological growth media. Phosphate salts (e.g., monosodium phosphate and its conjugate base disodium phosphate) are a common choice for buffering bacteriological media because they maintain the pH growth range of most bacteria. In solution, the phosphate buffer alters between a weak acid ($H_2PO_4^-$) and conjugate base (HPO_4^{2-}) to maintain H^+/OH^- equilibrium, as shown in the following equations:

$$H^+ + HPO_4^{2-} \rightarrow H_2PO^{4-}, \text{ and}$$

$$OH^- + H_2PO^{4-} \rightarrow HPO_4^{2-} + H_2O$$

Other buffer options also exist, but the principle of how they all work is the same, they stabilize pH by ensuring there is not a sharp overall change (increase or decrease) in H^+ concentration.

Classifying Prokaryotes by pH Growth Range

Although we can find bacteria living throughout the pH spectrum, most microbes grow best at a pH between 6.5 and 8.5. Like temperature and oxygen tolerance, pH tolerance is also a useful classification criterion. Within each microbe's survivable pH range are the **minimum, optimum,** and **maximum pH** for growth. The minimum pH is the lowest pH where growth is supported. The maximum pH is the highest pH where growth can occur. And the optimal is the preferred pH level for growth, the pH that supports the highest rate of cell division for a given microbe.

The three major classifications are:

1. **Acidophiles**: These organisms are adapted to grow well in environments of pH 1 (or less) to pH 5. We can find them in places like sulfur hot springs.
2. **Neutralophiles** (occasionally referred to as neutrophiles): Most microorganisms (and, by extension, most pathogens) prefer pH levels between 5 and 8.
3. **Alkaliphiles**: These organisms live at an alkaline (or basic) pH of about 9 to 11. These microbes can live in places like soda lakes that are enriched with sodium carbonate.

Clinical Applications

This is a qualitative procedure used to estimate the minimum, maximum, and optimum pH for growth of microbial species.

Understanding the cardinal pH ranges is important for culturing bacteria in the laboratory and it helps us understand the conditions that prevent or support the growth of certain pathogenic bacteria. For example, the acid environment in the stomach (pH of 1.5–3.5) is an important protection against a variety of potential pathogens that we may ingest in our food and/or water. Pathogens that cause gastrointestinal infections require specific adaptations to survive their passage through the stomach as well as through the acidic first segment of the small intestine. An example of such a pathogen is *Helicobacter pylori*, which causes stomach and duodenal ulcers. Given that this bacterium successfully infects the stomach, it seems counterintuitive to say this organism is neutralophilic. However, the bacterium doesn't survive for long in stomach acid. It instead employs temporary protections against stomach acid, measures that buy it enough time to burrow into the more neutral stomach mucosa to establish an infection.

Stomach pH is such an important first-line barrier to infection that regularly taking proton pump inhibitors (PPIs) for preventing acid reflux (e.g., drugs like Prilosec, Prevacid, Nexium, etc.) has been associated with increased susceptibility to certain gastrointestinal pathogens to include *Clostridioides difficile* (the causative agent of pseudomembranous colitis, "*C. diff*" infectious diarrhea), *Vibrio cholerae* (causative agent of cholera), and

Campylobacter jejuni (a leading cause of foodborne gastrointestinal infections). PPIs reduce H^+ concentrations in the stomach and thereby raise gastric pH, making it easier for certain pathogens to survive their brief transit through the stomach and successfully establish an infection in the intestinal tract.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ pH adjusted tryptic soy broth (TSB) (or nutrient broth, NB); 5 of each as follows: pH 2, pH 4, pH 6, pH 8, and pH 10 (25 total)
- ❑ Sterile transfer pipettes (4)
- ❑ Fresh broth cultures of these recommended bacteria:
  - ☐ *Alcaligenes faecalis* (recommend a BSL-1 strain ATCC 15554)
  - ☐ *Lactobacillus plantarum* or *Lactobacillus acidophilus*
  - ☐ *Lactococcus lactis*
  - ☐ *Staphylococcus saprophyticus*

Medium Recipe

pH-Adjusted Nutrient Broth (NB)

Beef extract	3.0 g
Peptone	5.0 g
Distilled or deionized water	1.0 L
NaOH or HCl as needed to adjust pH	

PROCEDURE

Today you will cultivate four organisms and observe how pH affects them. You will then classify the tested bacteria based on your results. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

1. **Obtain Your Media:** Working with your group, obtain five tubes of each pH broth—one of each pH per organism plus a control (25 tubes total).
2. **Label Your Tubes:** Label your tubes so that you have designated one broth for each organism plus one tube for an uninoculated control at each pH level (Fig. 21.2). Each student should choose one or two stock cultures from the options your instructor provides to inoculate. Divide the work load with your lab partner(s) as evenly as possible.
3. **Inoculate Your Media with the Designated Bacteria:** Mix each stock sample thoroughly, but be careful not to splash the culture into the tube cap. Then, using a sterile transfer pipette, inoculate each tube of media with 1 drop of organism (be consistent). Refer to Figure 21.2 for guidance on this. You may fill the pipette with about 1 mL of broth and deliver the drops with the same pipette to the tubes without flaming them. In your set of tubes, leave one tube uninoculated.

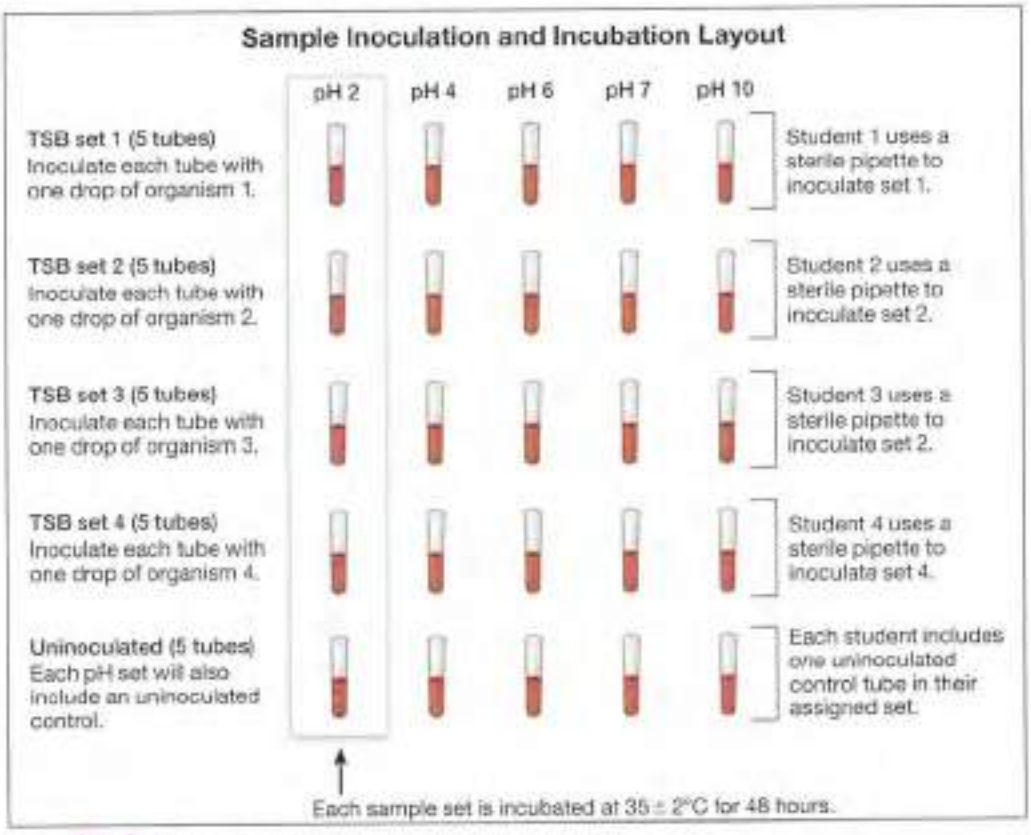

Sample Inoculation and Incubation Layout
pH 2
pH 4
pH 6
pH 7
pH 10
TSB set 1 (5 tubes)
Inoculate each tube with one drop of organism 1.
Student 1 uses a sterile pipette to inoculate set 1.
TSB set 2 (5 tubes)
Inoculate each tube with one drop of organism 2.
Student 2 uses a sterile pipette to inoculate set 2.
TSB set 3 (5 tubes)
Inoculate each tube with one drop of organism 3.
Student 3 uses a sterile pipette to inoculate set 2.
TSB set 4 (5 tubes)
Inoculate each tube with one drop of organism 4.
Student 4 uses a sterile pipette to inoculate set 4.
Uninoculated (5 tubes)
Each pH set will also include an uninoculated control.
Each student includes one uninoculated control tube in their assigned set.
Each sample set is incubated at 35 ± 2°C for 48 hours.

FIGURE **21.2** Broth Sample Organization and Inoculation Process

CAUTION!

- Your lab should have chemical solutions to clean up acid and base spills. If you spill any of the broths on the table or other inanimate objects, have a lab partner notify your instructor while you neutralize the spill with the appropriate solution available in your lab. For an acid spill, a sodium bicarbonate solution can be used. For a base spill, an acetic acid solution is appropriate. Be sure to know what neutralizing solutions are available in your laboratory and where they are located before you begin this laboratory exercise.
- If you spill on your skin, rinse thoroughly with water until your instructor gives you permission to stop. Do not try to neutralize acid and base spills on your skin.
- Before delivering the inoculum, insert the pipette well into the tube (but do not touch the broth).
- Do not spatter as you deliver the drop to each tube.
- Do not accidentally drip organism between tubes. If you do, then clean up the spill with proper decontamination approaches as your instructor directs.
- If you don't have enough organism left in the pipette to inoculate a tube, get more organism. You must not empty ("blow out") the pipette because you will create aerosols.
- Once finished, properly dispose of the pipette.

4 **Incubate the Samples:** Incubate all the samples at 35 ± 2°C for 48 hours.

5 **Clean up:** Save or dispose of the original cultures as your instructor directs.

→ Next Lab Session

1 **Obtain Your Samples:** Collect your cultures from the incubator (or wherever your instructor placed your samples).

2 **Clean the Outside of Each Broth Tube:** Use a clean tissue to wipe the glass of each broth tube and place each tube in a test-tube rack organized into groups by organism.

3 **Observe Your Broth Sample Data and Record the Results:** Gently mix each tube until you obtain uniform turbidity (cloudiness). Be careful not to splash the culture into the caps. Compare all tubes of a species to each other. Rate each as 0, 1, 2, or 3, according to its turbidity (0 is clear—the uninoculated tube—and 3 is the most turbid). Note your observations and results on the data sheet at the end of this lab.

4 **Analyze Your Broth Sample Data:** Use the data you recorded in Step 3 to make a graph. Use the graph paper provided with the data sheet at the end of this exercise. On your graph, plot the relative turbidity values versus pH for each organism. If you need help with graphing, see pages 10–12.

5 **Develop Conclusions Based on the Broth Growth Data:** On your graph, indicate the optimum pH for each bacterium studied. Then use brackets to indicate the pH range for each. (*Note:* This also indicates the minimum and maximum pH where you observed growth for a given organism.) Finally, use this data to develop a conclusion about the pH classification for each organism.

6 **Clean up:** Properly dispose of your samples as your instructor directs.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 21

DATA SHEET

The Effect of pH on Microbial Growth

During the Lab

1 For each organism, in the table provided record the turbidity (cloudiness) of the sample. The cloudier the sample, the more growth. Use your uninoculated control as a basis for your "0" point.

0 = no growth (completely clear sample)

1–2 = moderate growth

3 = abundant growth

Organism	pH 2	pH 4	pH 6	pH 8	pH 10	Classification

2 Plot relative turbidity (y-axis numeric values will be 0, 1, 2, 3) versus pH (x-axis) on the graph paper provided. If you need help with graphing, see pages 10–12.

3 Using the data in your graph, indicate the pH optimum for each organism on your graph and use brackets to designate the range (minimum to maximum) pH for each.

Lab Related Questions

1 From the following, what is the *best* conclusion about neutralophiles?

a. They thrive in relatively neutral environments.
b. They cannot survive an exposure to an acid.
c. They do not cause disease.
d. They are primarily pathogens.
e. They would not infect the stomach mucosa.

2 Which of the following describe(s) *Helicobacter pylori*? (Select all that apply.)

a. Pathogen
b. Acidophile
c. Neutralophile
d. Archaea
e. Causes stomach ulcers

3 In this experiment, _____ is the independent variable.

4 In this experiment, _____ is the dependent variable.

5 Match each term to its most appropriate descriptor(s).

_____ Alkaliphiles	a. Would grow at a pH of 3.
_____ Acidophiles	b. Would grow at a pH of 9.
_____ Neutralophiles	c. Include most human pathogens.
	d. Would grow at a pH of 6.5.

6 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ As the concentration of H^+ increases so does pH.

_____ A pH less than 7 is considered acidic.

_____ A pH greater than 7 is considered basic.

_____ The pH scale ranges from 1–14.

7 Choose the true statement(s) about buffers (select all that apply).

a. They stabilize solution pH.
b. They may be added to bacteriological growth media.
c. They are a combination of an acid and a base.
d. They raise pH when a base is added to a solution.
e. They lower pH when an acid is added to a solution.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 21
DATA SHEET
(continued)

Critical Thinking

1 As a hobby, you make pickles. Your cousin comes over and you offer them a taste of your latest batch of locally famous "never-cooked refrigerator sweet-and-sour cucumber dills." They turn down the offer and mention to you that the CDC (Centers for Disease Control and Prevention) just issued a warning that an outbreak of foodborne *Campylobacter jejuni* infections associated with cucumbers has been tearing through the region for the last month. The cases are all linked to cucumbers purchased at a local farmer's market, the same market where you buy all your cucumbers. Your cousin reminds you that the pickle batch you just completed was made using cucumbers that were harvested and sold during the time frame in which the infections occurred. Are your pickles, which soak in a pH 3.2 vinegar brine for at least a month, safe to eat? Explain your reasoning.

2 The "alkaline diet" has gained in popularity to improve general wellness, and even "prevent the growth of harmful bacteria." The premise of the diet is that certain foods raise your body's pH to an alkaline level (basic level) and that increase in pH is protective. Apply what you learned in this lab to discuss if this is a legitimate claim or wishful thinking.

SECTION

5

Media for the Selection and/or Differentiation of Bacteria

In This Section

EXERCISE 22 Mannitol Salt Agar (MSA) 229

EXERCISE 23 MacConkey (MAC) Agar 237

EXERCISE 24 Eosin Methylene Blue (EMB) Agar 245

EXERCISE 25 Blood Agar 253

— Lab Notes —

EXERCISE 22

Mannitol Salt Agar (MSA)

After doing this exercise, you should be able to:

1. Explain why MSA is a selective media.
2. Describe how MSA differentiates bacterial species from one another based on mannitol fermentation.
3. Discuss the formulation of MSA and state specifically which ingredients allow it to be selective versus differential.
4. Apply aseptic bacterial transfer and culture technique to set up an experiment that will assess how different bacteria grow on MSA.

Background

In Exercise 17, we learned about complex (or enriched) media versus defined (or synthetic) media. As a refresher, complex media contain extracts from, or digests of, plant and/or animal matter and are rich in nutrients to support the growth of fastidious microbes. In contrast, for defined media, each organic and inorganic ingredient is precisely known and quantified. In this exercise, we start our exploration of differential and selective media. Both complex and defined media can be modified in their ingredients to take on differential and/or selective properties.

Selective media contain ingredients that prevent the growth of certain bacteria. As their name states, they select for the growth of one class of microbe over another. **Differential media** include ingredients that allow us to visually distinguish (or differentiate) one group of bacteria from another based on metabolic features. In this lab, we explore how the medium **mannitol salt agar** (**MSA**) helps us select for certain bacteria that can tolerate salty environments. And for those bacteria that can grow in a salty environment, it allows us to differentiate bacteria based on their ability to ferment **mannitol** (a carbohydrate in the medium).

Selective and Differential Aspects of MSA

MSA is an enriched media, but because MSA contains 7.5% sodium chloride (NaCl), many bacteria cannot grow on it. The relatively high salt level exerts osmotic stress on cells (draws water out of cells that lack evolutionary adaptations to grow in salty environments). Thus, the media's salt level renders it selective for the growth of salt-tolerant bacteria such as members of the genera *Staphylococcus*, *Bacillus*, *Enterococcus*, *Micrococcus*, and others.

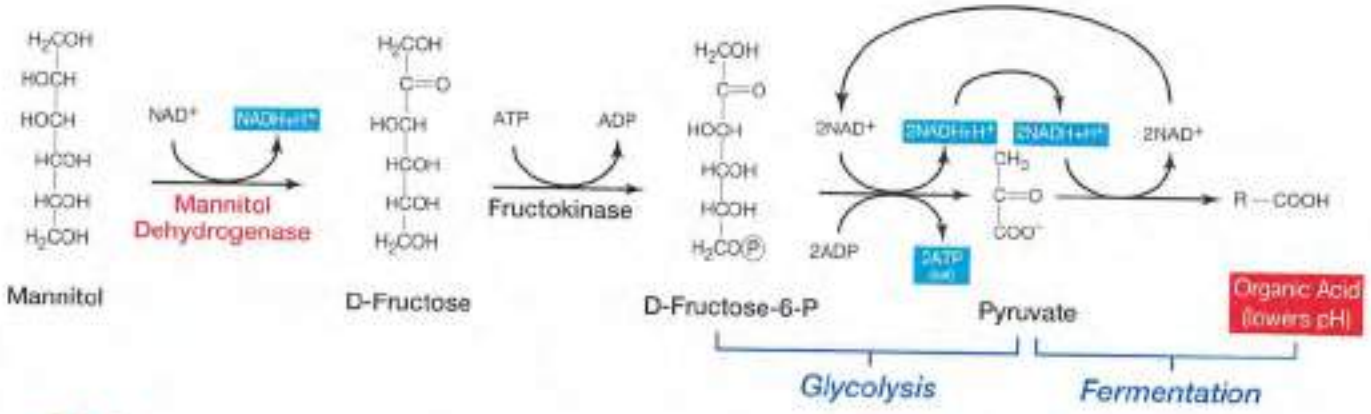
For those bacteria that will grow on MSA, we can then differentiate between those that can ferment the carbohydrate mannitol and those that cannot. This is easy to do through a visual inspection of the growth. Bacteria that can ferment mannitol release acids into the media and lower the local pH. The pH indicator in the media, **phenol red**, changes color in response to pH changes. Phenol red appears yellow below pH 6.8, red at pH 7.4 to 8.4, and pink at pH 8.4 and above. Consequently, the media around bacteria that can ferment mannitol appears yellow while the media around bacteria that can't ferment mannitol remains red/pink.

Differentiating *Staphylococcus* Species with MSA

As mentioned earlier, a number of bacterial genera can grow on MSA. However, this media is especially useful for differentiating *Staphylococcus* species from one another. Over 40 different *Staphylococcus* species have been characterized, and while most tend to grow well on MSA, not all of them ferment mannitol. The potential pathogen *Staphylococcus aureus* ferments mannitol, which produces acids and lowers the pH of the medium (Fig. 22.1). The result is formation of bright yellow colonies usually surrounded by a yellow halo (Fig. 22.2). For more information on fermentation, refer to Appendix A (Fig. A.6 and Table A.6).

It's important that you avoid four common misconceptions about MSA. First, while the genera of bacteria that can grow on MSA are usually Gram positive, this media is *not* selecting based on Gram property, it is selecting based on salt tolerance. Second, this media is *not* completely specific for isolating *Staphylococcus* species, even though that is how it's primarily used. Simply obtaining growth on this media is not enough for you to definitively conclude you are dealing with a *Staphylococcus* species. Third, this media is mainly employed as an isolation media, meaning that if we have a mixed sample (e.g., a swab of a skin wound) we can streak the sample onto this media to start the process to

see if *S. aureus*, a common skin wound pathogen, is present. Fourth, do not think that only *S. aureus* ferments mannitol. The truth is numerous (although not all) *Staphylococcus* species can ferment mannitol.



22.1 Mannitol Fermentation with Acid End Products • A key end product of mannitol fermentation is an organic acid (red box in the figure). That acid is detected by the phenol red pH indicator in MSA. For simplicity, only the relevant parts of glycolysis are shown. See Appendix A, Figure A.2 for details.

22.2 Mannitol Slat Agar (MSA) • Here you see two lines of inoculated bacteria. Each streak is of a different *Staphylococcus* species. The organism in the top streak fermented mannitol and therefore generated a yellow halo in the media. The bottom bacterium did not ferment mannitol, so the media remained red/pink.

Clinical Applications

Mannitol salt agar is mainly used to isolate and differentiate *Staphylococcus* species. These species are clinically significant; not only are they normal residents (normal microbiota) of the skin and mucosal surfaces that are near the skin (e.g., nasal passages, lower segment of the urethra, etc.), some are common pathogens.

S. aureus is arguably the most dangerous member of the *Staphylococcus* genus. It has unique **virulence factors** (features that make a pathogen better at establishing infection and advancing disease) that make it a versatile pathogen. It causes a wide range of infections such as sepsis, endocarditis pneumonia, toxic shock syndrome, and serious wound infections. However, other genus members are also problematic.

S. saprophyticus is one of the most common causes of urinary tract infections. *S. haemolyticus*, *S. hominis*, and *S. epidermidis* are common normal skin residents that don't typically cause disease in a healthy host, but they are problematic opportunistic pathogens, especially as causes of **health care-associated infections** (HAIs; also called **nosocomial infections**). For example, *S. haemolyticus*, *S. hominis*, and *S. epidermidis* are increasingly common causes of bacteremia (bacterial infection of the bloodstream). *S. epidermidis* is also increasingly responsible for orthopedic device-related infections. Adding to the clinical concern is the fact that we now find antibiotic resistant strains of all these bacterial species. Of these

five discussed species, *S. epidermidis* is the only one that doesn't usually ferment mannitol, the other four (*S. aureus*, *S. saprophyticus*, *S. haemolyticus*, and *S. hominis*) all ferment mannitol.

MATERIALS

- ☐ Lab coat
- ☐ Disposable gloves
- ☐ Safety glasses
- ☐ Mannitol salt agar (MSA) plate (1)
- ☐ Nutrient agar (NA) plate (1)
- ☐ Inoculating loop
- ☐ Fresh broth cultures of these recommended bacteria:
  - ☐ *Staphylococcus aureus* (this is a BSL-2 agent)
    - Alternatively, *Bacillus subtilis* (a BSL-1 agent) could be substituted for *S. aureus* if your lab is not approved for BSL-2 samples. This bacterium demonstrates the same metabolic features on MSA as *S. aureus*.
  - ☐ *Staphylococcus epidermidis*
  - ☐ *Escherichia coli*

Medium Recipes

Mannitol Salt Agar (MSA)

Beef extract	1.0 g
Peptone	10.0 g
Sodium chloride (NaCl)	75.0 g
D-Mannitol ($C_6H_{14}O_6$)	10.0 g
Phenol red	0.025 g
Agar	15.0 g
Distilled or deionized water	1.0 L

Adjust to pH 7.2–7.6 at 25°C

Nutrient Agar (NA)

Beef extract	3.0 g
Peptone	5.0 g
Agar	15.0 g
Distilled or deionized water	1.0 L

Adjust to pH 6.6–7.0 at 25°C

PROCEDURE

Today you will test how three organisms grow on nutrient agar versus on mannitol salt agar, a media that has selective and differential properties. MSA would normally be streaked for isolation, but in this lab, you will use a simple zigzag inoculation of pure cultures for the sole purpose of seeing what the different results look like.

1. **Obtain and Label Your Media:** Obtain your two plates of media (one MSA plate and one NA plate). Using a permanent marker, divide the bottom of each plate into three sectors. Label the base of each plate with the organisms' names (one organism in each plate section), your name, and the date. Use the same positions for each specimen on each plate.
2. **Inoculate Your Plates:** Gently mix each stock culture, being careful to not splash the culture into the tube's cap. Use an inoculating loop to perform a zigzag inoculation of each bacterium into their designated section on the plates. Inoculate three bacteria onto the MSA plate and the same three bacteria onto the NA plate. You will need to flame sterilize the loop between each organism so you do not cross-contaminate the samples. ***Caution!*** If you are not using all BSL-1 strains, then you must use BSL-2 safety practices. Follow your instructor's directions for this.
3. **Incubate the Samples:** Incubate all the samples at 35 ± 2°C for 48 hours.
4. **Clean up:** Save or dispose of the original cultures as your instructor directs.

→ Next Lab Session

1. **Obtain Your Samples:** Collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Observe Your Plates:** Examine and compare the plates for color and quality of growth. Note your observations and results on the data sheet at the end of this lab.
3. **Clean up:** Properly dispose of your samples as your instructor directs.

TABLE **22.1** Mannitol Salt Agar Results and Interpretations

Result	Interpretation	Presumptive ID
Poor growth (P) or no growth (N)	Organism's growth is inhibited by NaCl	Not a *Staphylococcus* species
Good growth (G)	Organism's growth is not inhibited by NaCl	*Staphylococcus* is possible, or some other bacterium known to grow well on MSA (e.g., a *Bacillus*, *Enterococcus*, *Micrococcus* species)
Yellow growth or halo (Y)	Organism produces acid from mannitol fermentation	Possible pathogenic *Staphylococcus aureus* (further testing needed to make this conclusion; many *Staphylococcus* species ferment mannitol and some *Staphylococcus* bacteria may ferment mannitol)
Red growth (no halo) (R)	Organism does not ferment mannitol	Organism other than *S. aureus*

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 22

DATA SHEET

Mannitol Salt Agar (MSA)

During the Lab

1 Record your results in the table provided, and use Table 22.1 to help you shape your interpretations.

Organism	Growth (N/P/G)		MSA Growth Color (Y/R)	Interpretation and Presumptive ID
	MSA	NA		

Lab Related Questions

1 You are studying a bacterial sample and you see that it failed to grow on MSA, but grew abundantly on nutrient agar. Which of the following is a reasonable conclusion about the bacterium?

a. It is not a *Staphylococcus* species.
b. It is *Escherichia coli*.
c. It is salt tolerant.
d. It is unable to ferment mannitol.
e. Its growth was inhibited by phenol red.

2 Choose the true statements about MSA (select all that apply).

a. It is an enriched media.
b. It is a selective media.
c. It is a differential media.
d. It only supports the growth of microbes that can use mannitol as a carbon source.
e. It supports the growth of pathogens.

3 If a bacterium grew on MSA and there is a _____ colored halo in the media, then it means _____ was fermented. In contrast, lack of a yellow halo around the bacterial growth means _____.

4 The role of phenol red in MSA is to:

a. Detect pH changes in the media.
b. Stabilize the media's pH.
c. Serve as a nutrient for the bacterial culture.
d. Prevent contamination.
e. Select for Gram-positive bacterial growth.

5 You are studying a bacterial sample and you see that it grew on MSA, but not on nutrient agar. What is the best conclusion?

a. The bacterium was an old stock sample and was unhealthy, which is why it didn't grow.
b. The bacterium is a fastidious microbe in terms of its nutrient requirements.
c. The bacterium requires an acidic pH to grow.
d. The bacterium is most likely a pathogen.
e. An error was made while inoculating the nutrient agar plate.

6 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ The only carbon source in MSA is mannitol.

_____ MSA is a defined media.

_____ MSA selects for growth based on bacterial salt tolerance.

_____ *Staphylococcus* species are not the only bacteria that can grow on MSA.

7 Choose the true statement(s) about *Staphylococcus* species (select all that apply).

a. They exist as normal human microbiota of the skin.
b. They include pathogens.
c. They are salt tolerant.
d. They include opportunistic pathogens.
e. They all ferment mannitol.

8 Which of the following is a misconception about MSA?

a. It selects for salt tolerant bacterial growth.
b. It selects for bacterial growth based on Gram property.
c. It is a complex media.
d. It will not support the growth of pathogens.
e. *Staphylococcus* species grow well on it.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 22
DATA SHEET
(continued)

Critical Thinking

A technician prepares several batches of MSA. Before a batch is cleared for use in laboratory applications, it must pass quality control (QC) analysis. For QC analysis, an MSA plate from each batch is inoculated with three different bacterial species. Organisms A and B should both grow on MSA; Organism C should not grow on MSA. Organism A can also ferment mannitol, but Organism B cannot. As a positive control, the same process (side-by-side inoculation of Organisms A, B, and C) is used to inoculate a nutrient agar (NA) plate. As a negative control, an uninoculated control is also included for each test run. The data for several separate MSA batches are presented in the following tables.

QC Testing Batch 1	Organism A	Organism B	Organism C	Uninoculated Plate
MSA	Abundant Growth Yellow Halo	Abundant Growth No Yellow Halo	Abundant Growth No Yellow Halo	No Growth
Nutrient Agar	Abundant Growth	Abundant Growth	Abundant Growth	No Growth

QC Testing Batch 2	Organism A	Organism B	Organism C	Uninoculated Plate
MSA	Abundant Growth Yellow Halo	Abundant Growth No Yellow Halo	No Growth	No Growth
Nutrient Agar	Abundant Growth	Abundant Growth	Abundant Growth	No Growth

QC Testing Batch 3	Organism A	Organism B	Organism C	Uninoculated Plate
MSA	Abundant Growth No Yellow Halo	No Growth	No Growth	Some Growth
Nutrient Agar	Abundant Growth	Abundant Growth	Abundant Growth	No Growth

Based on the data provided, answer the following questions:

1 Applying what you have learned about MSA, what criteria must be met for a batch to pass QC?

2 Which batch (or batches), if any, would you pass for use in the laboratory? Explain your reasoning.

3 The technician learned that one of the batches was prepared with a below normal amount of NaCl. Which batch would that most likely be? How do you know?

EXERCISE 23

MacConkey (MAC) Agar

After doing this exercise, you should be able to:

1. Explain why MAC agar is a selective media.
2. Describe how MAC agar differentiates bacterial species from one another based on lactose fermentation.
3. Discuss the formulation of MAC agar and state specifically which ingredients allow it to be selective versus differential.
4. Apply aseptic bacterial transfer and culture technique to set up an experiment that will assess how different bacteria grow on MAC agar.

Background

The family *Enterobacteriaceae* includes a diverse collection of Gram-negative, facultatively anaerobic, rod-shaped bacteria that do not form endospores. Members of this family are widespread through nature, with some of them existing as normal microbial residents of the intestinal tract in humans and other animals. These gut-associated prokaryotes are often called **enteric bacteria**. Among the family *Enterobacteriaceae*, we find the **coliforms**. Coliforms have the same general features of their *Enterobacteriaceae* cousins, but the distinct feature of coliforms is they readily ferment lactose to form acids. For more information about fermentation, refer to Appendix A (Fig. A.6 and Table A.6).

There are two main categories of coliforms. First, there are general **environmental coliforms**, which are abundant in soil and surface water, and most are not concerning from a clinical standpoint. Second, there are **fecal coliforms**, which as their name suggests are present in feces from humans and other warm-blooded animals. Fecal coliforms can include true pathogens, opportunistic pathogens, commensal organisms (organisms that do not hurt the host, but do not necessarily confer a benefit), and mutualistic microbes (organisms that confer a benefit on their host).

Isolating fecal coliform bacteria is useful because they are common indicators of fecal contamination in food and water. While most fecal coliforms are *not* pathogens, detecting them above a specific threshold in a sample suggests potential gut-associated pathogens may also be present. Because *Escherichia coli* is one of the most abundant fecal coliforms, it is considered the hallmark bacterium for fecal contamination. **MacConkey (MAC) agar** is one example of an enriched selective and differential growth media used for detecting coliforms (to include *E. coli*) in a sample.

Selective and Differential Aspects of MAC Agar

As discussed, coliforms are Gram negative and ferment lactose. Thus, to detect coliforms, MAC agar is formulated to select for Gram-negative bacteria and then differentiate this broad grouping of microbes based on their ability to ferment lactose. The bile salts and crystal violet in the media inhibit the growth of Gram-positive bacteria while allowing most Gram-negative bacteria to grow. In addition, lactose is present in the media along with a pH indicator called neutral red dye. This pH indicator is red at a pH of 6.8 and below, and yellow above pH 6.8. Therefore, organisms that ferment lactose to acidic end products lower the pH (Fig. 23.1) and their colonies turn a pink to red color. Sometimes a pink/red bile precipitate will also form in the agar due to the lowered pH. Lactose non-fermenters retain their normal color (which often is colorless) and some may impart a slightly yellow/tan tinge to the media (Fig. 23.2).

It's worth noting that since MAC agar was first developed in 1900, it has undergone a variety of formulations. The recipe we use here is formulated with a concentration of sodium chloride and bile salts that reduces swarming by bacteria like *Proteus* species (see Fig. 6.16, p. 96) that interfere with interpretation of results and isolation of other species. Another MAC agar recipe leaves out crystal violet and only uses bile salts as the ingredient that confers selectivity. This modified recipe allows certain Gram-positive bacteria such as *Enterococcus* and some species of *Staphylococcus* to grow. Because these bacteria ferment lactose, they appear pink on MAC agar.

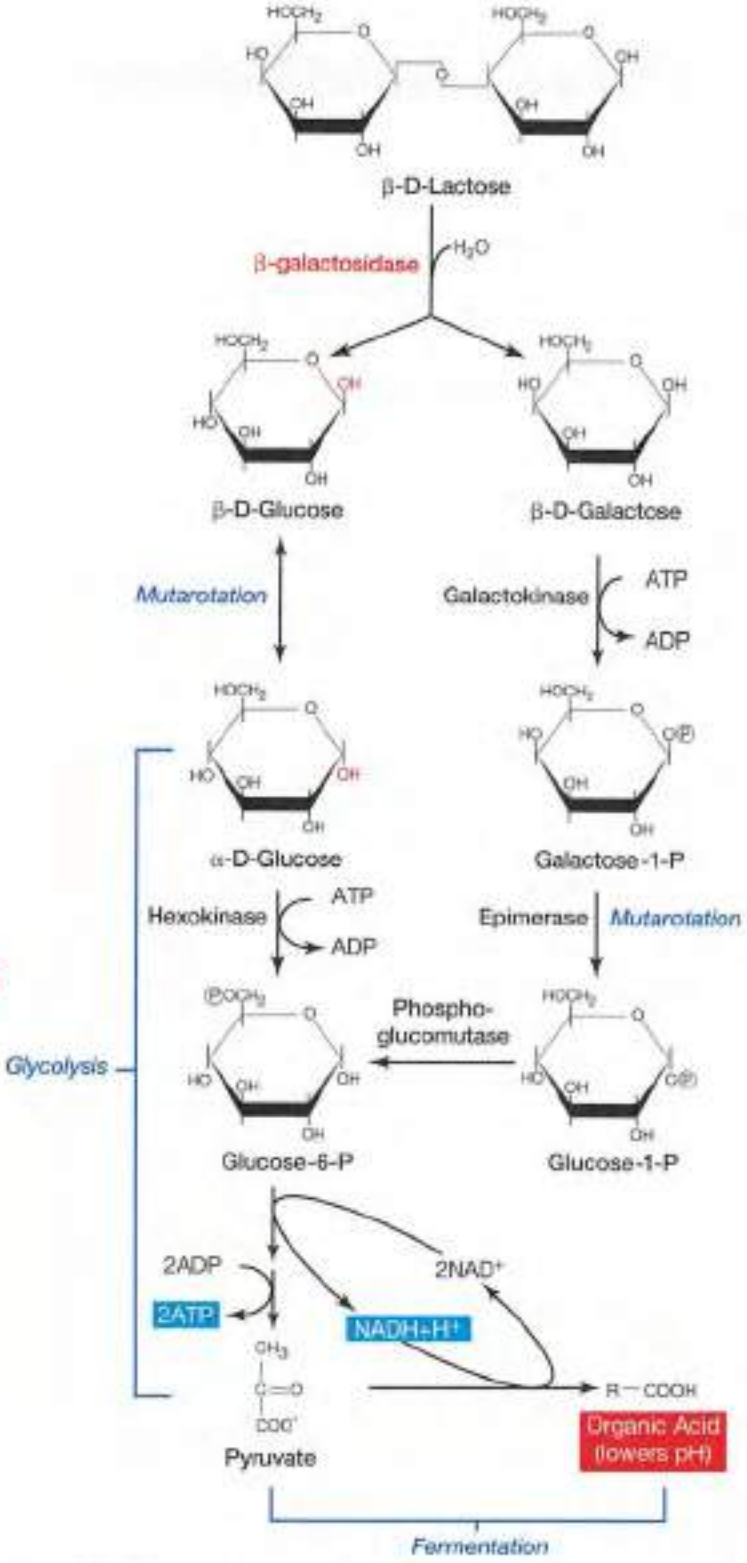


FIGURE 23.1 Lactose Fermentation with Acid End Products • A key end product of lactose fermentation is an organic acid (red box in the figure). That acid is detected by the neutral red pH indicator in MAC agar. For simplicity, only the relevant parts of glycolysis are shown. See Appendix A, Figure A.2 for details.

FIGURE 23.2 MacConkey (MAC) Agar • This MAC agar plate was inoculated with a Gram-positive coccus (center) and two members of *Enterobacteriaceae* (left and right). As expected, only the Gram-negative bacteria grew. The bacterium on the left (a coliform) fermented lactose and appears pinkish. The bacterium on the right (not a coliform), did not ferment lactose. It appears close to its natural color and has yellowed the medium.

Clinical Applications

Safety testing foods (e.g., milk, shellfish, and meats), drinking water, and recreational water (e.g., swimming pools and lakes) often involves checking for the presence of coliforms. This is because coliform bacteria are indicator organisms whose presence above a given threshold level suggest that certain enteric pathogens could be present. One example of an enteric pathogen that has caused numerous outbreaks by contaminating our food supply (e.g., beef products, various lettuces, and many other foods over the years) is *Escherichia coli* strain O157:H7. This strain is found in the gastrointestinal tract of cattle. In addition, the family *Enterobacteriaceae* includes a number of pathogens such as *Salmonella* serotype Typhi and *Salmonella* serotype Paratyphi, which causes typhoid fever and paratyphoid fever, respectively, as well as non-typhoidal *Salmonella* serotypes including Typhimurium and Enteritidis.

MATERIALS

- ☐ Lab coat
- ☐ Disposable gloves
- ☐ Safety glasses
- ☐ MacConkey (MAC) agar plate (1)
- ☐ Nutrient agar (NA) plate (1)
- ☐ Inoculating loop
- ☐ Fresh broth cultures of these recommended bacteria:
  - ☐ *Escherichia coli*
  - ☐ *Providencia stuartii*
  - ☐ *Staphylococcus epidermidis*

Medium Recipes

MacConkey (MAC) Agar

Pancreatic digest of gelatin	17.0 g
Pancreatic digest of casein ($C_{38}H_{57}N_9O_9$)	1.5 g
Peptic digest of animal tissue	1.5 g
Lactose ($C_{12}H_{22}O_{11}$)	10.0 g
Bile salts	1.5 g
Sodium chloride (NaCl)	5.0 g
Neutral red	0.03 g
Crystal violet	0.001 g
Agar	13.5 g
Distilled or deionized water	1.0 L

Adjust to pH 6.9–7.3 at 25°C

Nutrient Agar (NA)

Beef extract	3.0 g
Peptone	5.0 g
Agar	15.0 g
Distilled or deionized water	1.0 L

Adjust to pH 6.6–7.0 at 25°C

PROCEDURE

Today you will test how three organisms grow on nutrient agar versus on MacConkey (MAC) agar, a media that has selective and differential properties. MAC agar would normally be streaked for isolation, but in this lab, you will use a simple zigzag inoculation of pure cultures for the sole purpose of seeing what the different results look like.

1. **Obtain and Label Your Media:** Obtain your two plates of media (one MAC agar plate and one NA plate). Using a permanent marker, divide the bottom of each plate into three sectors. Label the base of each plate with the organisms' names (one organism in each plate section), your name, and the date. Use the same positions for each specimen on each plate.
2. **Inoculate Your Plates:** Gently mix each stock culture, being careful to not splash the culture into the tube's cap. Use an inoculating loop to perform a zigzag inoculation of each bacterium into their designated section on the plates. Inoculate three bacteria onto the MAC agar plate and the same three bacteria onto the NA plate. You will need to flame sterilize the loop between each organism so you do not cross-contaminate the samples.
3. **Incubate the Samples:** Incubate all the samples at 35 ± 2°C for 48 hours.
4. **Clean up:** Save or dispose of the original cultures as your instructor directs.

→ Next Lab Session

1. **Obtain Your Samples:** Collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Observe Your Plates:** Examine and compare the plates for color and quality of growth. Note your observations and results on the data sheet at the end of this lab.
3. **Clean up:** Properly dispose of your samples as your instructor directs.

TABLE **23.1** MacConkey Agar Results and Interpretations

Result	Interpretation	Presumptive ID
Poor growth (P) or no growth (N)	Organism is inhibited by crystal violet and/or bile	Gram-positive bacterium
Good growth (G)	Organism is not inhibited by crystal violet and/or bile	Gram-negative bacterium
Pink to red growth with or without bile precipitate (R)	Organism produces acid as a byproduct of lactose fermentation	Possible coliform bacterium
Growth is "colorless" (not red or pink) (C)	Organism does not ferment lactose	Unlikely to be a coliform bacterium

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 23
DATA SHEET

MacConkey (MAC) Agar

During the Lab

1 Record your results in the table provided, and use Table 23.1 to help you shape your interpretations.

Organism	Growth (N/P/G)		MAC Growth Color (R/C)	Interpretation and Presumptive ID
	MAC	NA		

Lab Related Questions

1 You are studying a bacterial sample and you see that it grew on MAC agar, but not on nutrient agar. What is the best conclusion?

a. The sample is a Gram-positive bacterium.
b. The sample is a Gram-negative bacterium.
c. The bacterium is most likely a pathogen.
d. There was an error made during the inoculation process.
e. The bacterium requires a neutral pH to grow.

2 Choose the true statements about MAC agar (select all that apply).

a. It is a defined media.
b. It is a selective media.
c. It supports the growth of pathogens.
d. It is a differential media.
e. Its recipe can be modified so that certain Gram-positive bacteria can grow on it.

3 The role of neutral red in MAC agar is to:

a. Serve as a nutrient for the bacterial culture.
b. Detect pH changes in the media.
c. Stabilize the media's pH.
d. Select for Gram-negative bacterial growth.
e. Select for Gram-positive bacterial growth.

4 If a bacterium grew on MAC agar and the bacteria takes on a _____ color, then it means _____ was fermented. In contrast, if the bacterial growth lacks coloration, it means _____.

5 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ MAC agar is used for water safety testing.

_____ Only pathogens grow on MAC agar.

_____ MAC agar selects for Gram-positive bacteria.

_____ Coliforms ferment lactose.

6 Choose the true statement(s) about *Enterobacteriaceae* (select all that apply).

a. It is a taxonomic family.
b. They include harmless bacteria.
c. They include Gram-positive bacteria.
d. They include fecal coliforms.
e. They include coccus-shaped bacteria.

7 Which of the following render MAC agar selective? (Select all that apply.)

a. Sodium chloride (NaCl)
b. Bile salts
c. Neutral red
d. Crystal violet
e. Lactose

8 Select the true statements about coliforms (select all that apply).

a. They include normal gut microbiota.
b. They include spore-forming bacteria.
c. They are Gram-negative bacteria.
d. They are only present if pathogens are present.
e. They include *Escherichia coli*.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 23
DATA SHEET
(continued)

Critical Thinking

1 Why do bile salts make an ideal ingredient in MAC agar to grow fecal coliforms over other bacteria?

2 Occasionally MAC agar is prepared as a broth medium, but such a format would not be useful for food safety or water quality testing. Explain why.

EXERCISE 24

Eosin Methylene Blue (EMB) Agar

After doing this exercise, you should be able to:

1. Explain why EMB agar is a selective media.
2. Describe how EMB agar differentiates bacterial species from one another based on lactose fermentation.
3. Discuss the formulation of EMB agar and state specifically which ingredients allow it to be selective versus differential.
4. Apply aseptic bacterial transfer and culture technique to set up an experiment that will assess how different bacteria grow on EMB agar.

Background

The media we explore in this lab is very similar to MacConkey (MAC) agar reviewed in Exercise 23. Like MAC agar, **eosin methylene blue (EMB) agar** is an enriched selective and differential growth media used for detecting members of the family *Enterobacteriaceae*. Recall that fecal coliforms are members of *Enterobacteriacea*. Because *Escherichia coli* is one of the most abundant fecal coliforms, it is considered the hallmark bacterium for fecal contamination of food and water.

Selective and Differential Aspects of EMB Agar

The gelatin in the media provides a rich nitrogen and organic carbon source. Lactose in the medium help us differentiate lactose fermenting coliforms, such as *Escherichia coli* and *Enterobacter aerogenes*, from pathogens such as *Proteus*, *Shigella*, and *Salmonella*, which do not ferment lactose. (See Fig. 23.1, p. 238, for an introduction to lactose fermentation.) The dyes eosin Y and methylene blue render the media selective and allow us to differentiate lactose fermenters from non-fermenters. Because the dyes inhibit the growth of most Gram-positive organisms (some *Enterococcus* and *Staphylococcus* species are exceptions), this media is selective for Gram-negative bacteria.

The capacity to differentiate between lactose fermenters and non-fermenters is due to how the dyes interact with the acidic end products that lactose fermenters produce. Based on the degree of lactose fermentation that occurs, these dyes will turn the bacterial growth a color ranging from a blackish/metallic-green to a pink/purple. A dark metallic-green growth is typical of vigorous lactose fermenters, like *Escherichia coli* (Fig. 24.1). Other, less vigorous lactose fermenters, such as *Enterobacter* or *Klebsiella* species, produce colonies that can range from pink to dark purple on the medium. Lactose non-fermenters typically retain their normal color or take on the coloration of the medium (Fig. 24.1).

FIGURE 24.1 Eosin Methylene Blue (EMB) Agar • This EMB agar plate was inoculated with a Gram-positive coccus (center) and two members of the Enterobacteriaceae, a coliform at the top and a noncoliform at the bottom. The Gram-positive coccus was inhibited by eosin and methylene blue dyes, but still grew enough to form a thin film on the agar. Vigorous lactose fermentation by the coliform gave its growth a shiny green appearance. The noncoliform bacterium did not ferment lactose, and therefore exhibits its regular coloration.

Clinical Applications

EMB agar is used to isolate fecal coliforms in food and dairy products as well as in environmental (especially water) samples. As mentioned in Exercise 23, this is because fecal coliform bacteria are indicator organisms whose presence above a given threshold level suggest that enteric pathogens such as certain *Escherichia coli* strains, *Salmonella*, *Shigella*, *Proteus*, *Klebsiella*, and *Yersinia*, may be present.

While pathogens within the *Enterobacteriaceae* family are important to consider in our food and water supply, these bacteria also cause a significant number of **nosocomial** or **health care-acquired infections (HAIs)** (infections that develop while a patient is receiving care in a health-care setting) and certain **community acquired infections** (infections that develop outside of a health-care setting). Compounding the importance of detecting

these bacteria in clinical samples is the fact that some *Enterobacteriaceae* family members are increasingly resistant to carbapenems along with many other antibiotics. These highly resistant bacteria are called **CRE strains**, which stands for carbapenem-resistant *Enterobacteriaceae*.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Eosin methylene blue (EMB) agar plate (1)
- ❑ Nutrient agar (NA) plate (1)
- ❑ Inoculating loop
- ❑ Fresh broth cultures of these recommended bacteria:
  - ❑ *Enterobacter aerogenes*
  - ❑ *Escherichia coli*
  - ❑ *Providencia stuartii*
  - ❑ *Staphylococcus epidermidis*

Medium Recipes

Eosin Methylene Blue (EMB) Agar (Levine)

Pancreatic digest of gelatin	10.0 g
Lactose ($C_{12}H_{22}O_{11}$)	10.0 g
Dipotassium phosphate (K_2HPO_4)	2.0 g
Methylene blue	0.065 g
Eosin Y	0.4 g
Agar	15.0 g
Distilled or deionized water	1.0 L

Adjust to pH 6.9–7.3 at 25°C

Nutrient Agar (NA)

Beef extract	3.0 g
Peptone	5.0 g
Agar	15.0 g
Distilled or deionized water	1.0 L

Adjust to pH 6.6–7.0 at 25°C

PROCEDURE

Today you will test how four organisms grow on nutrient agar versus on EMB agar, a media that has selective and differential properties. EMB agar would normally be streaked for isolation, but in this lab, you will use a simple zigzag inoculation of pure cultures for the sole purpose of seeing what the different results look like.

1. **Obtain and Label Your Media:** Obtain your two plates of media (one EMB agar plate and one NA plate). Using a permanent marker, divide the bottom of each plate into four sectors. Label the base of each plate with the organisms' names (one organism in each plate section), your name, and the date. Use the same positions for each specimen on each plate.
2. **Inoculate Your Plates:** Gently mix each stock culture, being careful to not splash the culture into the tube's cap. Use an inoculating loop to perform a zigzag inoculation of each bacterium into their designated section on the plates. Inoculate four bacteria onto the EMB agar plate and the same four bacteria onto the NA plate. You will need to flame sterilize the loop between each organism so you do not cross-contaminate the samples.
3. **Incubate the Samples:** Incubate all the samples at 35 ± 2°C for 48 hours.
4. **Clean up:** Save or dispose of the original cultures as your instructor directs.

→ Next Lab Session

1. **Obtain Your Samples:** Collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Observe Your Plates:** Examine and compare the plates for color and quality of growth. Note your observations and results on the data sheet at the end of this lab.
3. **Clean up:** Properly dispose of your samples as your instructor directs.

TABLE **24.1** Eosin Methylene Blue Agar Results and Interpretations

Result	Interpretation	Presumptive ID
Poor growth (P) or no growth (N)	Organism is inhibited by eosin and/or methylene blue	Most likely a Gram-positive bacterium
Good growth (G)	Organism is not inhibited by eosin and/or methylene blue	Most likely a Gram-negative bacterium
Growth is pink and mucoid (PI)	Organism produces acid as a byproduct of lactose fermentation	Possible coliform bacterium
Growth is "dark" (purple to black, with or without green metallic sheen) (D)	Organism produces acid as a byproduct of lactose fermentation	Probable coliform bacterium
Growth is "colorless" (not purple, green, red, or pink) (C)	Organism does not ferment lactose	Unlikely to be a coliform bacterium

— Lab Notes —

5

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 24

DATA SHEET

Eosin Methylene Blue (EMB) Agar

During the Lab

1 Record your results in the table provided, and use Table 24.1 to help you shape your interpretations.

Organism	Growth (N/P/G)		EMB Growth Color (Pi/D/C)	Interpretation and Presumptive ID
	EMB	NA		

Lab Related Questions

1 You are studying a bacterial sample and you see that it grew on NA but not on EMB agar. What is the best conclusion?

a. The bacterium is likely Gram positive.
b. The bacterium does not use lactose as a carbon source.
c. The bacterium is a pathogen.
d. The bacterium was from an older culture, so it did not grow as expected.
e. The dye eosin was left out of the EMB agar when it was prepared.

2 Choose the true statements about EMB agar (select all that apply).

a. This is a defined medium.
b. This is a selective medium.
c. This is an enriched medium.
d. This is a differential medium.
e. This medium detects a bacterium's ability to ferment glucose.

3 What is the role of methylene blue in EMB agar? (Select all that apply.)

a. It allows bacteria to ferment lactose.
b. It serves as a nutrient source in EMB agar.
c. It acidifies the pH of the medium when lactose is fermented.
d. It is important for differentiating lactose fermenters from non-fermenters.
e. It has a role in inhibiting Gram-positive bacteria from growing.

4 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ Bacteria that ferment lactose will always grow on EMB agar.

_____ Gram-positive bacteria appear green when grown on EMB agar.

_____ EMB agar selects for Gram-negative bacteria.

_____ Coliforms do not typically grow on EMB agar.

5 Choose the true statements about the lactose in EMB agar (select all that apply).

a. Lactose fermenters may appear pink.
b. Lactose fermenters may appear dark green/blackish.
c. Most coliforms can ferment lactose.
d. EMB agar only allows us to assess lactose fermentation ability for Gram-positive bacteria.
e. Bacteria will only grow on EMB agar if they can use lactose as a carbon source.

6 What is the role of eosin Y in EMB agar? (Select all that apply.)

a. It selects for the growth of Gram-positive bacteria.
b. Bacteria that grow on EMB agar use eosin Y as a carbon source.
c. It stabilizes the media's pH.
d. It helps us visually differentiate lactose fermenters from non-fermenters.
e. It inhibits the growth of most Gram-positive bacteria.

7 Why wouldn't it be advisable to compare growth of the organisms on each plate to each other? (Select all that apply.)

a. NA medium is selective for Gram-positive bacteria, while EMB agar is selective for Gram-negative bacteria.
b. NA medium is nutrient deficient and will not grow fastidious bacteria, while EMB agar is enriched and therefore will support the growth of fastidious bacteria.
c. We can expect anything that grows well on NA to also grow on EMB agar.
d. We can expect bacteria that grow well on EMB agar will also grow on NA.
e. Barring experimental process errors, a lack of growth on NA should be accompanied by a lack of growth on EMB agar.

8 If a bacterium grew on EMB agar and the bacteria takes on a dark-green color, then it means _____.

In contrast, if the bacterial growth lacks coloration, it means _____. A pink bacterial growth coloration suggests _____.

9 *Enterobacteriaceae* family members are important to detect because (select all that apply):

a. They can contaminate foods.
b. They can cause HAIs.
c. They can cause community-acquired infections.
d. They indicate fecal contamination of water.
e. They are all pathogens.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 24
DATA SHEET
(continued)

Critical Thinking

1 Would removing eosin Y and/or methylene blue from EMB agar alter the medium's sensitivity or specificity? Explain your answer. (See pp. 9–10, "A Word About Experimental Design," for assistance with these terms.)

2 Suppose a mistake was made while preparing a batch of EMB agar and the starting pH is 7.6 instead of 6.9–7.3. Would that affect the medium's sensitivity or specificity? Explain your answer. (See pp. 9–10, "A Word About Experimental Design," for assistance with these terms.)

— Lab Notes —

EXERCISE 25

Blood Agar

After doing this exercise, you should be able to:

1. Describe the formulation of blood agar.
2. Explain how blood agar differentiates bacterial species from one another.
3. Discuss the forms of hemolysis.
4. State how blood agar can be clinically useful.
5. Apply aseptic bacterial transfer and culture technique to set up an experiment that will assess the hemolytic properties of select bacteria by growing them on blood agar.

CAUTION!

The U.S. Centers for Disease Control and Prevention cautions, "Either nonpathogenic or attenuated bacterial strains should be used when possible, especially in teaching laboratories." This lab exercise can either be performed with samples collected via throat swab or you may use known bacterial samples. Both protocols are described herein. Before deciding to use throat cultures as the inoculum source, faculty should check institutional policies regarding cultivating unknown bacteria from human subjects. Anytime such sampling is performed, BSL-2 agents that are *not* attenuated could be isolated. Faculty are strongly cautioned to review their institution's policies on students handling such samples.

Background

Blood agar is an enriched medium that includes 5% blood (usually sheep blood) in a tryptic soy agar base. Its rich composition allows for the growth of fastidious organisms, while also allowing for the differentiation of bacteria—especially certain Gram-positive cocci such as *Streptococcus*, *Staphylococcus*, and *Enterococcus* species—based on the degree to which they conduct **hemolysis** (break down RBCs).

The three major types of hemolysis are: **α-hemolysis** (alpha hemolysis), **β-hemolysis** (beta hemolysis), and **γ-hemolysis** (gamma hemolysis). Although all three of these include the word hemolysis, only one—β-hemolysis—completely breaks down RBCs and hemoglobin as indicated by a clearing of the medium around bacterial colonies (Fig. 25.1). In contrast, α-hemolysis, which is often described as a partial breakdown of RBCs, involves the oxidation of hemoglobin inside RBCs to methemoglobin.[1] This produces an olive-greenish discoloration of the blood agar around the colonies in reflected light (Fig. 25.2). Finally, in γ-hemolysis the RBCs are not broken down at all, nor is hemoglobin affected, so there is no change to the medium (Fig. 25.3).

But why would certain bacteria even conduct hemolysis? Well, iron is usually an essential nutrient for bacterial growth; however, it is not typically circulating freely in the human body. Instead it is bound in proteins like hemoglobin and other iron-containing proteins. Therefore, pathogens that conduct β-hemolysis may have a survival advantage due to their ability to completely break down RBCs and hemoglobin to obtain iron. While most pathogenic *Streptococcus* species happen to be α- or β-hemolytic, you should not assume that a bacterium must be α- or β-hemolytic to be pathogenic. Hemolytic activity is just one of many virulence factors that bacteria may have and there are plenty of non-hemolytic bacteria in nature that cause disease.

FIGURE 25.1 β-Hemolysis • *Streptococcus pyogenes* demonstrates β-hemolysis. The clearing around the growth is a result of complete lysis of red blood cells in the medium. For this photograph, the plate was illuminated with transmitted light.

[1] Hemoglobin is the oxygen-carrying molecule in RBCs. It contains an iron atom (Fe), which can lose electrons (undergo an oxidation to become Fe^{3+}) or gain electrons (undergo a reduction to become Fe^{2+}). Hemoglobin's iron atom must be in the Fe^{2+} state to bind oxygen. In methemoglobin, which cannot bind oxygen, the iron exists in the Fe^{3+} state.

FIGURE 25.2 α-Hemolysis • This is a streak plate of *Streptococcus pneumoniae* demonstrating α-hemolysis. The greenish zone around the colonies results from incomplete lysis of red blood cells in the medium. For this photograph, the plate was illuminated with transmitted light, so the coloration appears more yellow-green than olive-green.

FIGURE 25.3 γ-Hemolysis • This streak plate of *Staphylococcus epidermidis* on a sheep blood agar shows that red blood cells in the medium are not lysed.

Hemolytic Bacteria

Many bacterial pathogens make **hemolysins**, which are specialized proteins that can affect RBCs, hemoglobin, and other cells such as white blood cells.

Here we focus on the hemolysins that certain members of the genus *Streptococcus* make, **streptolysins**. They come in two forms: type O and type S. **Streptolysin O** is oxygen labile (unstable in the presence of oxygen) and therefore works best under anaerobic conditions. **Streptolysin S** is oxygen stable, but also works best under anaerobic conditions. Therefore, it is useful to the **streak-stab technique** to detect streptolysin activity for bacteria inoculated onto blood agar. In this technique, a blood agar plate is streaked on the agar surface and then the agar is stabbed with the loop at the end of the streak pattern. The stab encourages streptolysin activity because of the reduced oxygen concentration of the subsurface environment. The result may be α-hemolysis on the surface, but β-hemolysis in the stab (Fig. 25.4).

FIGURE 25.4 Aerobic Versus Anaerobic Hemolysis • An unidentified throat culture isolate demonstrates α-hemolysis when growing on the surface, but β-hemolysis beneath the surface surrounding the stab (**arrow**). This results from production of an oxygen-labile hemolysin.

Clinical Applications

Blood agar is commonly used to differentiate pathogenic *Streptococcus* species from non-pathogenic species of this diverse genus of Gram-positive cocci. In humans, streptococcal pathogens primarily infect the respiratory tract, skin, and bloodstream. One of the most common streptococcal pathogens identified in clinical samples is *S. pyogenes*. This bacterium is typically β-hemolytic and causes a wide range of infections to include streptococcal pharyngitis (strep throat), scarlet fever, streptococcal toxic shock syndrome, necrotizing fasciitis (flesh-eating bacteria), superficial skin infections (impetigo), and deeper skin infections (cellulitis). Blood agar remains an important means of detecting *S. pyogenes* in clinical samples, to include definitively diagnosing streptococcal pharyngitis. Other noteworthy streptococcal pathogens include *S. pneumonia* (bacterial pneumonia), and *S. mutans* (dental caries).

MATERIALS

- Lab coat
- Disposable gloves
- Safety glasses
- Sheep blood agar plate (1) (commercially available, TSA containing 5% sheep blood)
- Inoculating loop
- If a sample will not be collected by throat swab, then provide fresh broth cultures of these recommended bacteria:
  - *Streptococcus pyogenes* (BSL-2)
  - *Escherichia coli* (BSL-1 strains are standard)
  - *Streptococcus mutans* (BSL-1 strains are standard)

Medium Recipes

5% Sheep Blood Agar (TSA + 5% Sheep Blood)

Infusion from beef heart (solids)	2.0 g
Pancreatic digest of casein ($C_{58}H_{97}N_9O_9$)	13.0 g
Sodium chloride (NaCl)	5.0 g
Yeast extract	5.0 g
Agar	15.0 g
Defibrinated sheep blood	50.0 mL
Distilled or deionized water	1.0 L

Adjust to pH 7.1–7.5 at 25°C

PROCEDURE

Faculty may approach this lab differently depending on institutional safety regulations and the availability of BSL-2 graded lab facilities. As such, you will either inoculate stock cultures onto a blood agar plate to observe the various forms of hemolysis, or you may perform a throat swab and culture bacteria from that specimen. Follow whichever procedure your instructor decides to use. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

OPTION 1: Throat Culture Procedure

1. **Obtain and Label Your Media:** Obtain a blood agar plate. Use a permanent marker to mark the bottom of the plastic plate so it is divided into four equal quadrants. Then label the plate with your name, the date, and "throat culture."
2. **Perform a Throat Swab:** Have your lab partner (also appropriately protected) obtain a sterile cotton-tipped swab and follow the procedure in Appendix B, page 475, to obtain the specimen from your throat.
3. **Use the Swab to Inoculate the First Quadrant of the Blood Agar:** For this procedure, you will inoculate the agar using a zigzag inoculation combined with streak plating (see Ex. 4, p. 73, to review the process). Immediately inoculate the collected specimen into the first quadrant of the blood agar plate. Once you've inoculated the first plate quadrant, dispose of the swab into an approved biological waste container.
4. **Use an Inoculating Loop to Inoculate the Remaining Quadrants of the Blood Agar:** Use your inoculating loop to inoculate the remaining three plate sections. (Again, see Ex. 4, p. 69–71, to review the process.)
5. **Incubate the Samples:** Because of the potential for cultivating a pathogen from a throat swab specimen, tape down the plate lid once you have finished inoculating the plate. This will reduce the chance of the plate being accidentally opened. Invert the plate and incubate your sample at 35 ± 2°C for 24 hours; remove the plate to the refrigerator if you cannot examine it at 24 hours.
6. **Clean up:** Disinfect your work surface and thoroughly wash your hands.

→ Next Lab Session

1. **Obtain Your Samples:** Collect your cultures from the incubator (or wherever your instructor placed your samples). Do not open your plate until your instructor has seen it and given permission to do so.
2. **Observe Your Plates:** Holding your plate up to a light or using transmitted light from a light box/colony counter may help you better detect color changes and clearing around the colonies. Compare your observations to the information in Table 25.1 and note your observations and results on the data sheet at the end of this lab.
3. **Clean up:** Properly dispose of your samples as your instructor directs.

OPTION 2: Stock Culture Procedure

1. **Obtain and Label Your Media:** Obtain a blood agar plate. Use a permanent marker to mark the bottom of the plastic plate so it is divided into three equal sections. Label one section as *S. pyogenes*, another as *E. coli*, and the third as *S. mutans*.
2. **Inoculate the Blood Agar with the Streak-Stab Technique:** Use an inoculating loop to perform a zigzag inoculation of each sample into its designated plate section. At the tail end of the zigzag surface

inoculation, use the loop to make a small stab/incision into the agar as shown in Figure 25.4. This will allow you to see the difference in hemolytic action for each bacterium under reduced oxygen conditions. ***Note:*** Do not interrupt your streaked surface with this stab inoculation; instead stab at the tail end of the streak pattern.

3. **Incubate the Samples:** Invert the plate and incubate your sample at 35 ± 2°C for 24 hours; remove the plate to the refrigerator if you cannot examine it at 24 hours.
4. **Clean up:** Disinfect your work surface and thoroughly wash your hands.

→ Next Lab Session

1. **Obtain Your Samples:** Collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Observe Your Plates:** Holding your plate up to a light or using transmitted light from a light box/colony counter may help you better detect color changes and clearing around the colonies. Compare your observations to the information in Table 25.1 and note your observations and results on the data sheet at the end of this lab.
3. **Clean up:** Properly dispose of your samples as your instructor directs.

TABLE **25.1** Blood Agar Test Results and Interpretations

Result	Interpretation	Symbol
Clearing around growth	Organism hemolyzes RBCs completely	β-hemolysis
Greening around growth	Organism partially hemolyzes RBCs/hemoglobin oxidized to methemoglobin	α-hemolysis
No change in the medium	Organism does not hemolyze RBCs	No hemolysis (also called γ-hemolysis)

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 25
DATA SHEET

Blood Agar

During the Lab

OPTION 1: Throat Culture Procedure

Observe your plate and find four different colonies growing on the blood agar surface (try to find a diversity of hemolysis reactions). Based on your observations, complete the following table. Refer to Table 25.1 for assistance with interpreting hemolytic property, and Figure 6.6 on page 91 when reporting colony morphology information.

	Colony Morphology and Agar Appearance	Hemolysis Result (α, β, γ)	Interpretation
Colony 1			
Colony 2			
Colony 3			
Colony 4			

OPTION 2: Stock Culture Procedure

Observe each of the three cultured bacteria and complete the following table. Refer to Table 25.1 for assistance with interpreting hemolytic property.

Organism	Agar Appearance Surface Inoculation	Agar Appearance Stab Inoculation	Surface Inoculation Hemolysis Result (α, β, γ)	Stab Inoculation Hemolysis Result (α, β, γ)	Interpretation

Lab Related Questions

1 Select the false statement(s) about blood agar (select all that apply).

a. It is a selective medium.
b. It is an enriched medium.
c. It is a differential medium.
d. It is useful for growing fastidious bacteria.
e. It inhibits the growth of Gram-negative bacteria.

2 Which of the following is not caused by *Streptococcus pyogenes*?

a. Streptococcal toxic shock syndrome
b. Necrotizing fasciitis
c. Dental caries
d. Streptococcal pharyngitis
e. Impetigo

3 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ Methemoglobin is an oxidized form of hemoglobin.

_____ *Streptococcus* species are differentiated from one another on blood agar based on Gram property.

_____ Blood agar is usually prepared with bovine (cow) blood.

_____ Only pathogens grow on blood agar.

4 Choose the mismatched pair(s) (select all that apply).

a. *Streptococcus*; Gram-negative
b. *S. pyogenes*; β-hemolytic
c. Streptolysin O; oxygen labile
d. Streptolysin S; oxygen labile
e. γ-hemolysis; lack of hemolysis

5 Which of the following is not a reason for stabbing blood agar? (Select all that apply.)

a. It helps to detect streptolysin O activity.
b. It helps to detect streptolysin S activity.
c. It helps to isolate bacterial species from one another.
d. It tells us if we have a mixed culture.
e. It allows *Streptococcus* species to grow.

6 A bacterium with a _____ coloration around colonies is said to conduct _____ hemolysis, which is described as a partial lysis of the RBCs in the medium. In contrast, _____ generates a clearing around the bacteria due to _____.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 25
DATA SHEET
(continued)

7 Select the false statement(s) about hemolysis (select all that apply).

a. γ-hemolysis is a virulence factor.

b. All pathogens conduct α- or β-hemolysis.

c. Hemolysis only occurs under anaerobic conditions.

d. α-hemolysis must occur before β-hemolysis.

e. *S. pyogenes* performs β-hemolysis.

Critical Thinking

1 You are studying a newly emerging pathogen and determine the organism exhibits β-hemolysis when growing on the surface of blood agar, but exhibits γ-hemolysis when inoculated via a "stab" into blood agar. Based only on this observation, what may you conclude about this bacterium's unique hemolysin(s)?

2 Although streptolysins tend to be most active under decreased oxygen levels, throat culture samples are not analyzed using a stab inoculation. Explain why this is logical.

— Lab Notes —

SECTION

6

Biochemical Tests for Identifying Bacterial Isolates

In This Section

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— Lab Notes —

EXERCISE 26

Oxidation-Fermentation (O-F) Test

After doing this exercise, you should be able to:

1. State what O-F media can reveal about a bacterial species.
2. Discuss the possible interpretations for various O-F test results.
3. Describe the formulation of oxidation-fermentation (O-F) media.
4. Explain how O-F media is used in a clinical microbiology laboratory.
5. Apply aseptic bacterial transfer and culture technique to set up an experiment that will assess the oxidative-fermentative metabolic features of select bacteria by growing them on O-F media.

Background

The **Oxidation-Fermentation (O-F) test** (Hugh and Leifson's O-F media) differentiates bacteria—especially certain Gram-negative bacteria—based on how they metabolize carbohydrates. Specifically, this test reveals if a bacterial species uses aerobic oxidative respiration and/or fermentation to breakdown a given sugar.

Oxidative Respiration Versus Fermentation

Oxidative respiration involves stripping electrons off nutrients (via glycolysis, an intermediate step, and the Krebs cycle) and shuttling those harvested electrons through an electron transport chain to a final electron acceptor to generate ATP. In **aerobic oxidative respiration**, oxygen serves as the final electron acceptor in the electron transport chain and is therefore an absolute requirement for this pathway. In addition to making ATP, aerobic oxidative respiration also produces carbon dioxide (CO_2) and water (H_2O).

The O-F test is formulated to detect the mildly acidic end products that oxidative respiration generates. In addition, this test detects if a bacterium ferments a specific sugar to form acidic end products. Unlike aerobic oxidative respiration, **fermentation** does not use an electron transport chain and does not require oxygen to make ATP. Bacteria are metabolically versatile, so some species perform oxidative respiration and fermentation, choosing which is best based on the availability of oxygen.

How the O-F Test Works

Hugh and Leifson's O-F media contains a single sugar—usually glucose (dextrose)—as the carbohydrate source. In addition, the media has a high sugar-to-peptone ratio, which renders it especially useful for detecting the weakly acidic end products made during oxidative respiration. This is because the alkaline (basic) products produced when bacteria digest protein will not be present in quantities that readily neutralize weak acids that oxidative metabolism produces. Bromothymol blue dye serves as the pH indicator in the media. It appears yellow at a mildly acidic pH of 6.0, green at a near neutral pH of 7.1, and blue at the slightly alkaline pH of 7.6.

Before inoculation, the tubes of O-F media are placed in a boiling-water bath to drive off dissolved oxygen that may be in the media. Then, once the tubes of media have cooled, a bacterial sample is inoculated into two tubes; one tube will be set up to maintain anaerobic conditions, while the other will promote aerobic conditions. For the anaerobic tube, immediately following inoculation, the media's surface is sealed off with a layer of mineral oil. This prevents oxygen diffusion into the media and thereby prevents aerobic oxidative metabolism while allowing fermentation (Fig. 26.1). For the second tube, the media's surface is not sealed off with oil, allowing oxygen to diffuse through the semisolid media to support aerobic oxidative respiration (although fermentation is not prevented). Following incubation, the samples are observed to determine if there was a color change from green (the original media color) to yellow (Fig. 26.2).

FIGURE **26.1** **Adding the Sterile Mineral Oil Layer** • Tip the tube slightly to one side, and gently add sterile mineral oil to a depth of 3–4 mm. Be sure to use a sterile pipette for each tube.

Table 26.1 provides interpretations for the possible color outcomes. If the test organism ferments glucose, then *both* the sealed and unsealed media should turn yellow. If the organism performs *only* oxidative metabolism of glucose, then only the unsealed media turns yellow. Note that oxidative metabolism and fermentation both cause the unsealed media to turn yellow. Thus, while O-F media can reveal if an organism strictly uses oxidative metabolism to breakdown glucose, it does not reveal if an organism can metabolize glucose with oxidative *and* fermentation processes or strictly via fermentation. This is an important test limitation to recognize.

TABLE **26.1** Oxidation-Fermentation (O-F) Test Results and Interpretations •

Sealed (anaerobic tube)	Unsealed (aerobic tube)	Interpretation	Symbol
Green or blue	Any amount of yellow	Oxidation only	O
Green or blue	Green or blue	No sugar metabolism; organism is nonsaccharolytic (cannot metabolize the sugar in the medium)	N
Yellow throughout	Yellow throughout	Oxidation and fermentation; or could be fermentation only	O-F or F[1]
Slightly yellow at the top	Slightly yellow at the top	Slow oxidation and slow fermentation; or could be slow fermentation only	O-F or F[1]

[1] The test does not differentiate between species that are strictly fermentative and species that conduct both fermentation *and* oxidative metabolism of the sugar. Therefore, when both tubes are yellow, the result is recorded as (O-F) or (F).

	Sealed Media Appearance	Unsealed Media Appearance	
Oxidative only	Green	Yellow	
Fermentation only	Yellow	Yellow	The test results for these two scenarios appear identical; thus, the O-F test does not allow us to differentiate between these two scenarios.
Oxidative and Fermentation	Yellow	Yellow	

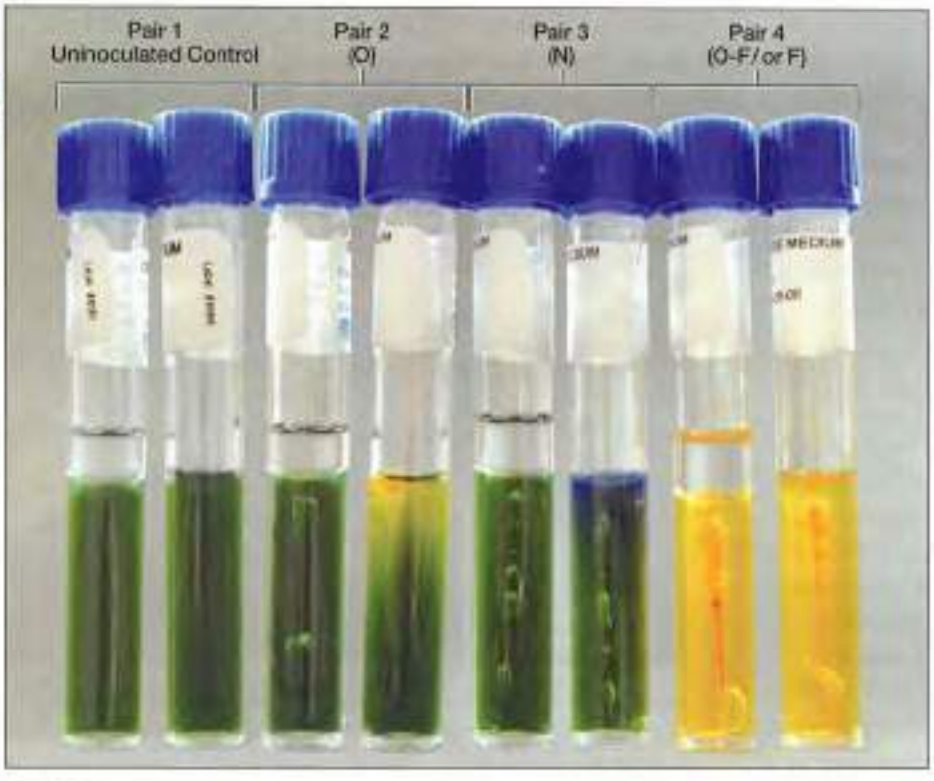


FIGURE **26.2** **Oxidation-Fermentation (O-F) Test Results** • These tubes illustrate three possible results for the O-F test. **Pair 1** is an uninoculated control for color comparison. **Pair 2** was inoculated with a bacterium that only conducts oxidative metabolism (**O**) of glucose. **Pair 3** was inoculated with an organism incapable of utilizing glucose. Note the blue color in the upper portion of the unsealed media suggesting that the organism is both nonsaccharolytic (**N**) and strictly aerobic. **Pair 4** was inoculated with an organism capable of both oxidative and fermentative, or perhaps just fermentative, utilization of glucose (**O-F** or **F**). Recall the O-F test does not differentiate bacteria that use oxidative and fermentative processes from those that only use fermentative processes to metabolize the sugar in the media.

Clinical Applications

Determining a bacterium's identity is a key diagnostic tool. Many biochemical tests exist to help microbiologists accomplish this task. The O-F test is used to differentiate bacteria based on their ability to oxidize or ferment specific sugars. It allows presumptive separation of the fermentative *Enterobacteriaceae* (a large family that includes important pathogens such as *Escherichia coli* and *Salmonella*) from the strictly oxidative *Pseudomonas* (a genus that includes *P. aeruginosa*, which causes wound infections and swimmer's ear, otitis externa).

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ O-F glucose tubes (8)
- ❑ Sterile mineral oil
- ❑ Sterile transfer pipettes
- ❑ Fresh agar slant cultures of these recommended organisms:
  - ❑ *Alcaligenes faecalis* (subspecies *faecalis*) (recommend a BSL-1 strain ATCC 15554)
  - ❑ *Escherichia coli*
  - ❑ *Kocuria rosea*

Medium Recipe

Hugh and Leifson's O-F Medium with Glucose

Pancreatic digest of casein ($C_{18}H_{27}N_5O_9$)	2.0 g
Sodium chloride (NaCl)	5.0 g
Dipotassium phosphate (K_2HPO_4)	0.3 g
Agar	2.5 g
Bromothymol blue	0.08 g
Glucose ($C_6H_{12}O_6$)	10.0 g
Distilled or deionized water	1.0 L

Adjust to pH = 6.6–7.0 at 25°C

PROCEDURE

Eight O-F media tubes will be used for this exercise, six for inoculation (two for each organism) and two for controls. One of each pair will receive a mineral oil overlay to maintain an anaerobic environment and force fermentation if the organism is capable of doing so. For best results, use a heavy inoculum and several stabs for each tube. Use only sterile pipettes to add the mineral oil. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

1. **Obtain and Label Your Media:** Obtain eight O-F tubes. Label six (in three pairs) with the names of the organisms, your group name, and the date. Label the last pair "control."
2. **Inoculate the Media:** Using an inoculating needle, stab inoculate each pair (aside from the uninoculated control tubes) with the appropriate test organism to a depth of about 1 cm from the bottom of the agar. Most bacteria grow slowly in O-F media; therefore, to have observable results by your next class session, you will need to inoculate the tubes heavily. To introduce a heavy inoculum, simply repeat the inoculation process two additional times per tube so each tube is ultimately inoculated with three times the normal amount of inoculum.
3. **Add Mineral Oil:** Using a different sterile transfer pipette for each organism overlay one tube of each pair (including the controls) with 3–4 mm of sterile mineral oil (Fig. 26.1).
4. **Incubate the Samples:** Incubate all tubes at 35±2°C for 48 hours.
5. **Clean up:** Dispose of stock cultures as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

→ **Next Lab Session**

1. **Obtain Your Samples:** Collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Observe Your Samples:** Examine the tubes for color changes. Be sure to compare them to the controls before making a determination. Record your results in the table provided on the data sheet at the end of this lab.
3. **Clean up:** Properly dispose of your samples as your instructor directs.

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 26
DATA SHEET

Oxidation-Fermentation (O-F) Test

During the Lab

1 Observe your tubes, and use Table 26.1 to help you complete the following table.

Organism	Color Results		Symbol	Interpretation
	Sealed	Unsealed		
Uninoculated controls				

Lab Related Questions

1 Select the true statement(s) about O-F media (select all that apply).

a. It is a differential medium.
b. It is a selective medium.
c. It contains methylene blue as a pH indicator.
d. The test is inoculated in pairs so that the media can be sealed off from atmospheric oxygen in one case, but not the other.
e. The media can differentiate between bacteria that use both oxidative and fermentation processes from those that only conduct fermentation.

2 Why is mineral oil added in the O-F test?

a. To serve as a nutrient source.
b. To keep potential pathogens from becoming airborne.
c. To remove oxygen from the media.
d. To help oxygen diffuse through the media.
e. To maintain an anaerobic environment.

3 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ If a bacterium carries out oxidative metabolism, it cannot also carry out fermentation.

_____ O-F media is designed to detect the alkaline byproducts of fermentation.

_____ O-F media provides a single carbohydrate source.

_____ The O-F test is selective for bacteria that conduct fermentation versus aerobic oxidative respiration.

4 Facultative anaerobes, such as members of the family *Enterobacteriaceae*, are capable of both aerobic respiration and fermentation. What color results would you expect for such organisms when grown in O-F glucose media?

a. The sealed media should turn yellow and the unsealed media should remain green.
b. The sealed media should remain green and the unsealed media should turn yellow.
c. The sealed tube should turn yellow and the unsealed tube should turn blue.
d. Both the sealed and unsealed media should turn yellow.
e. Both the sealed and unsealed tubes should remain green.

5 Assume you were studying a newly discovered bacterium that is aerotolerant (can grow in the presence of oxygen) and performs anaerobic respiration where a certain amino acid that is common in proteins serves as the final electron acceptor. What observations would you expect for such a specimen?

a. The sealed media should turn yellow and the unsealed media should remain green.
b. The sealed media should remain green and the unsealed media should turn yellow.
c. The sealed tube should turn yellow and the unsealed tube should turn blue.
d. Both the sealed and unsealed media should turn yellow.
e. Both the sealed and unsealed tubes should remain green.

6 In the O-F test performed in this exercise, _____ served as the carbohydrate source while _____ served as a protein source.

Critical Thinking

1 Why is it necessary to use two controls (uninoculated control and a nonsaccharolytic sample) rather than just one? What are the specific purposes of each? Be thorough in your answer.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 26
DATA SHEET
(continued)

2 Suppose you perform this exercise and upon making your observations, you notice the unsealed, uninoculated control contained slight yellowing at the top. Suppose further that Pair 1 showed complete yellowing of both tubes and Pairs 2 and 3 showed slight yellowing of the unsealed tube. Assuming all other tubes were green, what conclusions could you safely make?

— Lab Notes —

EXERCISE 27

Phenol Red Fermentation Broth

After doing this exercise, you should be able to:

1. State what growing a bacterium in phenol red fermentation broth can reveal.
2. Discuss the possible interpretations for various phenol red fermentation broth results.
3. Describe how phenol red broth is formulated to provide metabolic information about a bacterial species.
4. Explain how phenol red fermentation broth can be useful in a clinical microbiology laboratory.
5. Apply aseptic bacterial transfer and culture technique to set up an experiment that will assess the fermentative metabolic features of select bacteria by growing them in phenol red fermentation broth.

Background

Carbohydrate fermentation is a generalized biochemical process in which a carbohydrate, usually a sugar, is metabolized to make ATP without the use of oxidative phosphorylation. Recall that oxidative phosphorylation can be aerobic (the electron transport chain requires oxygen as a final electron acceptor) or anaerobic (the electron transport chain does not require oxygen as a final electron acceptor). Most microbes are metabolically flexible, so depending upon available nutrients and other environmental conditions they may conduct fermentation or perform oxidative phosphorylation (aerobic or anaerobic).

Monosaccharides and **disaccharides** are some of the most commonly fermented carbohydrates. Monosaccharides are the simplest carbohydrates, examples include the sugars glucose (dextrose), fructose (levulose), and galactose. Monosaccharides are sometimes called *simple sugars* because they do not need to be broken down into smaller carbohydrate units before entering metabolic pathways. In contrast, disaccharides are sugars made up of two monosaccharides. Disaccharides must be broken down (**hydrolyzed**) into their simple sugar components before entering metabolic pathways. Two common disaccharides are lactose and sucrose. The initial step in metabolically processing these sugars involves hydrolyzing them into their monosaccharide components: lactose is hydrolyzed into glucose and galactose whereas sucrose is split to release glucose and fructose. Thus, when an organism is described as a "lactose fermenter," it means the organism splits the disaccharide lactose into the monosaccharides glucose and galactose and then ferments those monosaccharides.

In microbiology we frequently use the term **fermenter**; unless expressly stated otherwise, this term should be assumed to include the initial hydrolysis of a carbohydrate into monosaccharide subunits followed by fermentation pathways that further metabolize the simple sugars. *Note:* Unlike aerobic oxidative phosphorylation, fermentation does not require oxygen to occur. However, this point should not lead you to assume that bacteria only conduct fermentation under anaerobic conditions. In this laboratory exercise your samples are grown in aerobic conditions.

In most cases, carbohydrate fermentation begins with simple sugars entering glycolysis, which makes pyruvate as a product. (Instead of glycolysis, some organisms use alternative pathways, such as the Entner-Doudoroff pathway [Fig. A.3, p. 470], to make pyruvate.) In fermentation, the resulting pyruvic acid is further metabolized to make a variety of organic acids, alcohols, and hydrogen or carbon dioxide gas. Fermentation reactions are diverse and the exact end products made depend on what enzymes the organism has available to perform a subset of reactions. Refer to Fig. 27.1 and Fig. A.6 for more information about fermentation pathways.

How Phenol Red Broth Works

Phenol red broth is a differential medium that can reveal if a bacterium ferments a specific carbohydrate. In addition to a protein source (pancreatic digest of casein), the media contains one of several carbohydrates (e.g., glucose, lactose, sucrose), the pH indicator phenol red, and a Durham tube to detect gas formation.

After inoculation and incubation, the phenol red broth is observed for two things:

Color Change

The media's color after incubation indicates if the sugar in the media was fermented. This is due to the media's pH indicator, phenol red, which appears yellow below pH 6.8, pink/magenta above pH 7.4, and red in between. Carbohydrate fermentation often produces acidic end products that lower the media's pH below the neutral range thereby turning the medium yellow (Fig. 27.2). If the organism did not ferment the sugar and instead digested protein, then the media may appear pink due

to the alkaline (basic) end products associated with protein metabolism. You can expect red media for uninoculated samples and for samples containing bacteria that either made neutral end products or failed to react at all.

Gas Production

Some organisms produce gas (often carbon dioxide or hydrogen gas) as a fermentation byproduct. If this occurs, the gas becomes trapped in the inverted Durham tube

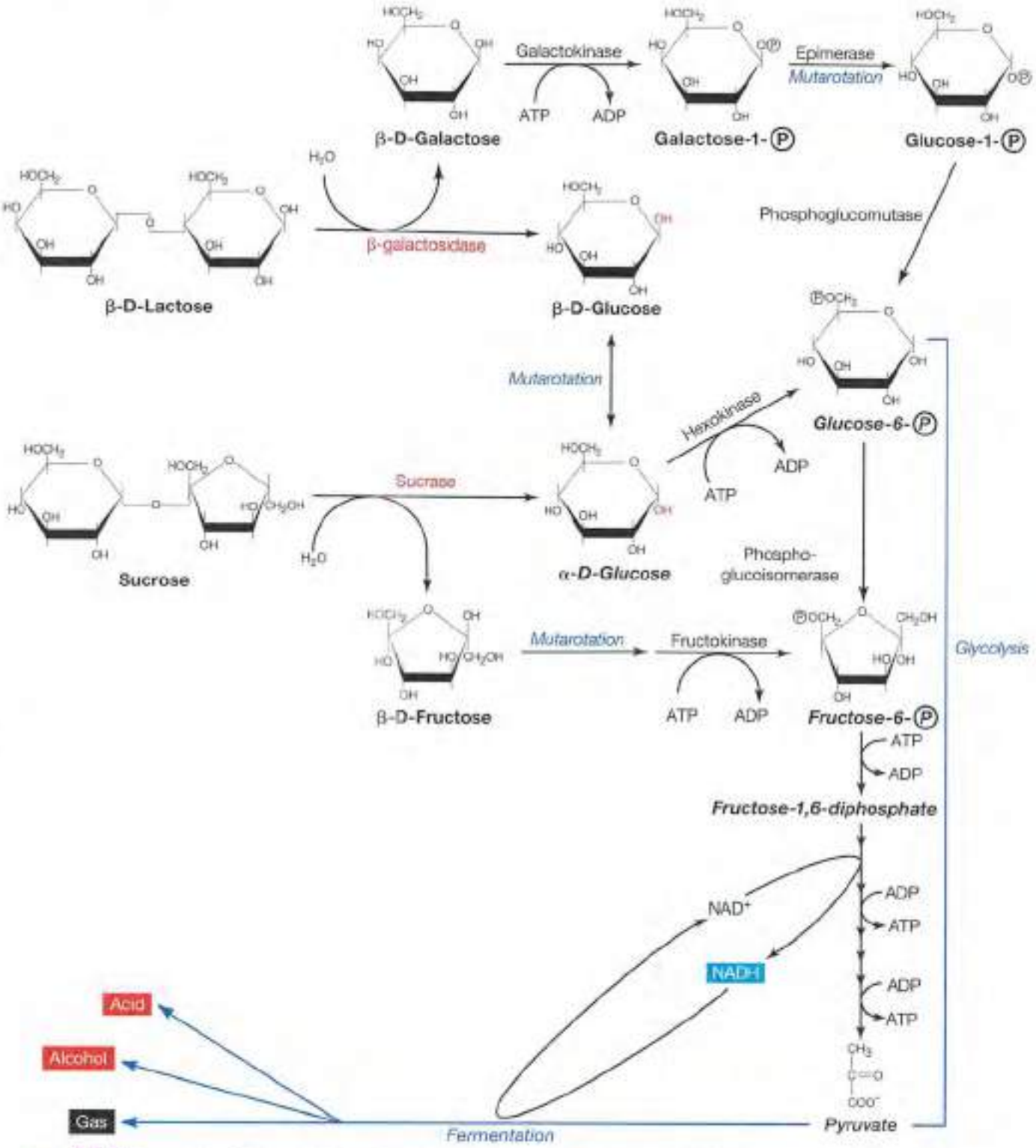


FIGURE **27.1** **Fermentation of Glucose and the Disaccharides Lactose and Sucrose** • Enzymes hydrolyze the disaccharides lactose and sucrose to release monosaccharides. These monosaccharides are then funneled into glycolysis as the initial stage in fermentation. The enzyme β-galactosidase hydrolyzes lactose into glucose and galactose whereas sucrase hydrolyzes sucrose to release glucose and fructose.

and a bubble is evident. Note that multiple tiny, pinpoint-sized bubbles in a Durham tube are typically artifacts of media preparation and/or due to samples being mixed. If gas is truly made, the bubble is comparatively large and easy to see.

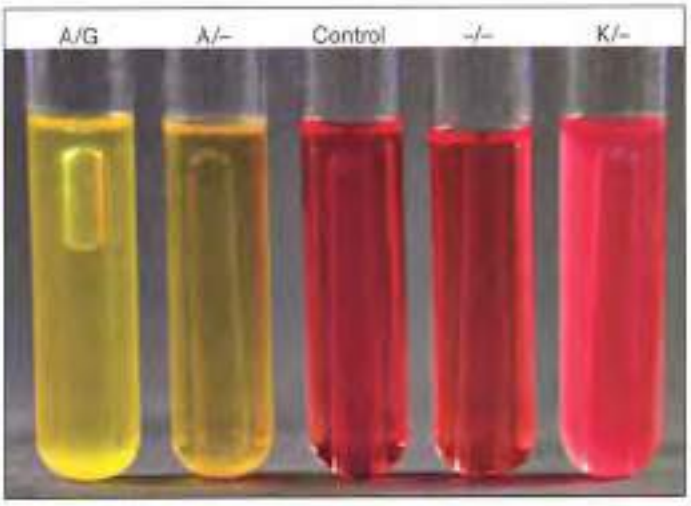


FIGURE **27.2** **Phenol Red Glucose Fermentation Broth Results** • These five phenol red glucose broths demonstrate various results. From left to right: **A/G** = fermentation with acid and gas production; **A/–** = fermentation with acid formation but without gas production; **Control** = uninoculated tube; **–/–** = no reaction (no fermentation reaction and no peptone breakdown)/lack of gas formation; **K/–** = no fermentation, instead proteins were metabolized to form basic end products/no gas produced.

TABLE **27.1** Phenol Red Fermentation Broth Test Results and Interpretations •

Result (list broth color first, then note if a bubble is present or absent)	Interpretation	Symbol
Yellow broth; bubble in Durham tube	The carbohydrate (insert sugar's name) was fermented and acidic end products were formed; gas was also produced as a byproduct. (***Tip:*** Per Fig. 27.2, if lactose was fermented, you can note that your test bacterium makes the enzyme β-galactosidase; if sucrose was fermented, you can state your test bacterium makes sucrase.)	A/G
Yellow broth; no bubble in Durham tube	The carbohydrate (insert sugar's name) was fermented and acidic end products were formed; gas was also produced as a byproduct. (***Tip:*** Per Fig. 27.2, if lactose was fermented, you can note that your test bacterium makes the enzyme β-galactosidase; if sucrose was fermented, you can state your test bacterium makes sucrase.)	A/–
Red broth; no bubble in Durham tube	The carbohydrate (insert sugar's name) was not fermented and gas was not produced.	–/–
Pink broth; no bubble in Durham tube	Degradation of peptone occurred to form alkaline end products, but the carbohydrate (insert sugar's name) was not fermented; gas was not produced.	K/–
Orange broth; no bubble in Durham tube	This is often noted as a negative test result for fermentation. However, it does suggest acidic products are present, albeit at very low levels since the media's pH remained above 6.8. Follow your instructor's directions on how they want you to note this result. They may want you to incubate the sample longer and/or note the result as inconclusive.	

Symbol Key:

A = Acidic end products (broth appears yellow due to fermentation reaction)

K = Alkaline end products (broth appears pink due to peptone breakdown; no fermentation of the given carbohydrate)

G = Gas bubble present in Durham tube

–/– = No reaction (no fermentation reaction and no peptone breakdown/lack of gas formation)

Clinical Applications

A variety of biochemical test procedures help clinical microbiologists identify pathogenic microbes in a patient sample (e.g., urine sample, throat swab, blood sample, etc.), phenol red medium is one such biochemical test. It helps us determine the fermentation profile for a specimen and is most commonly used to differentiate members of *Enterobacteriaceae* from other Gram-negative rods. It also allows us to distinguish between Gram-positive fermenters, such as *Streptococcus* and *Lactobacillus* species. Some *Streptococcus* species, such as *S. mutans* are prolific fermenters that make acidic end products that cause dental caries (cavities). The vaginal microbiome includes *Lactobacillus* species, which ferment glycogen (a polymer of glucose) and thereby establish an acidic pH in this tissue. The acidic vaginal pH inhibits the growth of a variety of potential pathogens to include the yeast *Candida albicans*, which causes vulvovaginal candidiasis (yeast infections).

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Phenol red glucose broths with Durham tubes (4)[1]
- ❑ Phenol red base broths (media without a carbohydrate added) with Durham tubes (4, optional)
- ❑ Phenol red broth containing other carbohydrates (e.g., lactose or sucrose) with Durham tubes (optional)
- ❑ Fresh agar slant cultures of these recommended organisms:
  - ❑ *Alcaligenes faecalis* (subspecies *faecalis*) (recommend a BSL-1 strain ATCC 15554)
  - ❑ *Escherichia coli*
  - ❑ *Providencia stuartii*
  - ❑ *Streptococcus gallolyticus*; recommended if using additional phenol red broths (optional)

Medium Recipe

Phenol Red (Carbohydrate) Broth[2]

Pancreatic digest of casein ($C_{38}H_{57}N_9O_9$)	10.0 g
Sodium chloride (NaCl)	5.0 g
Carbohydrate (one only: glucose, lactose, sucrose, etc.)	5.0 g
Phenol red	0.018 g
Distilled or deionized water	1.0 L
Adjust to pH = 7.1–7.5 at 25°C	

[1] This lab exercise can also be performed using purple broths, which differ only slightly in ingredient composition, but use bromocresol purple as the pH indicator. Bromocresol purple turns yellow at pH 5.2 and is purple above pH 6.8.

[2] This formulation without carbohydrate is phenol red base broth.

PROCEDURE

Today you will inoculate phenol red glucose broths with three organisms to determine their fermentation characteristics. Your instructor may direct you to use phenol red broths containing other carbohydrates (e.g., lactose, sucrose, etc.) in addition to, or in place of, phenol red glucose broth. As a reminder, include an uninoculated control of each medium for color comparison. After your samples have incubated, use Table 27.1 as a guide for interpreting and recording your results.

1. **Obtain and Label Your Media:** Obtain four phenol red glucose broth tubes. Label each with your name, the medium name, and the date. Label the first three tubes with the names of the bacteria you will inoculate into each tube and label the fourth tube "uninoculated control." If your instructor wants you to use phenol red broth with other carbohydrates, you'll label those tubes in the same manner.
2. **Inoculate the Media:** Use an inoculating loop to inoculate the broth in each of your three experimental tubes with a test organism. Leave the fourth tube uninoculated.
3. **Incubate the Samples:** Incubate your samples at 35±2°C for 48 hours.
4. **Clean up:** Dispose of stock cultures as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

→ Next Lab Session

1. **Obtain Your Samples:** Collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Observe Your Samples:** Using the uninoculated control as a color comparison and Table 27.1 as a guide, examine your samples and enter the results on the data sheet at the end of this lab exercise. When indicating the various reactions, use the standard symbols as shown, with the acid reading first, followed by a slash and then the gas reading.
3. **Clean up:** Properly dispose of your samples as your instructor directs.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 27
DATA SHEET

Phenol Red Fermentation Broth

During the Lab

1 Observe your tubes, and use Table 27.1 to write up your results in the following table.

	Phenol Red Results (list color first, then gas information)				
	Base Broth (optional)	Glucose	Lactose (optional)	Sucrose (optional)	Interpretation
Uninoculated control					Base broth (optional):
					Glucose:
					Lactose (optional):
					Sucrose (optional):
Organism Name	**Base Broth (optional)**	**Glucose**	**Lactose (optional)**	**Sucrose (optional)**	**Interpretation**
					Base broth (optional):
					Glucose:
					Lactose (optional):
					Sucrose (optional):

Organism Name	Base Broth (optional)	Glucose	Lactose (optional)	Sucrose (optional)	Interpretation
					Base broth (optional):
					Glucose:
					Lactose (optional):
					Sucrose (optional):

Organism Name	Base Broth (optional)	Glucose	Lactose (optional)	Sucrose (optional)	Interpretation
					Base broth (optional):
					Glucose:
					Lactose (optional):
					Sucrose (optional):

Organism Name	Base Broth (optional)	Glucose	Lactose (optional)	Sucrose (optional)	Interpretation
					Base broth (optional):
					Glucose:
					Lactose (optional):
					Sucrose (optional):

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 27
DATA SHEET
(continued)

Lab Related Questions

1 Choose the false statement(s) about the phenol red test (select all that apply).

a. It can detect if an organism ferments a specific sugar.
b. It is a differential medium.
c. It is a selective medium.
d. It is inoculated with a mixed culture.
e. It may contain multiple sugars in the same sample tube.

2 The purpose of the Durham tube in the media is:

a. To stabilize the media's pH.
b. To capture gas made as a fermentation byproduct.
c. To remove oxygen from the media.
d. To help oxygen diffuse through the media.
e. To detect the media's pH.

3 Choose the true statement(s) about fermentation (select all that apply).

a. If a fermentation reaction occurs, the phenol red media should turn yellow.
b. All fermentation reactions produce gas as a byproduct.
c. Fermentation reactions do not require oxygen.
d. Glycolysis is the first stage of fermentation.
e. Fermentation can only occur under anaerobic conditions.

4 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ Monosaccharides must be converted to disaccharides before being metabolized by fermentation pathways.

_____ Before it can be fermented, lactose must be split into simple sugars by the enzyme glucosidase.

_____ Sucrose is a disaccharide made up of fructose and glucose.

_____ A bacterium may conduct oxidative phosphorylation as well as fermentation.

5 The enzyme _____ is required to split sucrose into monosaccharides before fermentation can occur.

6 Assume you conduct a phenol red glucose test on an unknown bacterium and after incubation you obtain an A/– result. What can you reasonably conclude about the bacterium? (Select all that apply.)

a. The bacterium ferments the sugar glucose.
b. The bacterium makes alkaline metabolic byproducts.
c. The bacterium made acidic end products.
d. The bacterium does not make gas as a fermentation byproduct.
e. There was an absence of gas as well as an absence of a color change.

Critical Thinking

1 Should you anticipate a bubble in the Durham tube if the media is red? Explain your reasoning.

2 Suppose you perform this exercise and upon making your observation, you notice the media *outside* the Durham tube is red, while the media *inside* the Durham tube is yellow. What conclusions could you make? Be sure to explain your reasoning.

EXERCISE 28

Methyl Red and Voges-Proskauer (MR-VP) Tests

After doing this exercise, you should be able to:

1. Describe the formulation of MR-VP media.
2. State how the MR and VP tests are each performed and interpreted.
3. Explain what the MR and VP tests each reveal about a bacteria species.
4. State why MR-VP tests are clinically useful.
5. Apply aseptic bacterial transfer and culture technique to set up an experiment that will assess if bacteria perform mixed acid fermentation or butanediol fermentation.

Background

Recall that fermentation reactions are metabolic processes that harvest energy from a nutrient source (often a sugar) without the use of an electron transport chain (without oxidative phosphorylation). While fermentation is an anaerobic process and therefore does not require oxygen to occur, that does not mean fermentation exclusively occurs when oxygen is absent; many microbes conduct fermentation when oxygen is present. Think of it like this: a flashlight may be especially useful during a power outage, but a power outage is not necessary for you to use a flashlight.

Fermentation reactions are extremely diverse, so much so that even when the same sugar is fermented (e.g., glucose), different end products can be made depending upon the pathway the bacterium uses. Consequently, determining the nature of a fermentation reaction's end products can help us differentiate bacterial species from one another. The **methyl red and Voges-Proskauer Tests** (MR-VP) help us differentiate bacterial species based on the fermentation pathway used to metabolize glucose.

Mixed Acid and Butanediol Fermentation Pathways

Fermentation pathways are diverse, but the MR-VP test differentiates between only two fermentation processes: mixed acid fermentation and butanediol fermentation (also called 2,3-butanediol fermentation).

In **mixed acid fermentation**, the pyruvate made in glycolysis is metabolized to form a variety of end products such as lactate, formate, acetate, succinate, ethanol, carbon dioxide (CO_2), and hydrogen gas (H_2) (Fig. 28.1). Among these end products, those with the suffix "ate" are acids—formate, acetate, succinate—that can stably lower (acidify) the MR-VP media's pH.

In **butanediol fermentation**, pyruvate is metabolized to form many of the same products seen for mixed acid fermentation, but in far smaller quantities. Instead, most of the end products are 2,3-butanediol and ethanol, which are neutral (Fig. 28.2). Because butanediol fermentation mainly generates neutral end products, the MR-VP media's pH will be closer to neutral than if mixed acid fermentation occurred (Fig. 28.3).

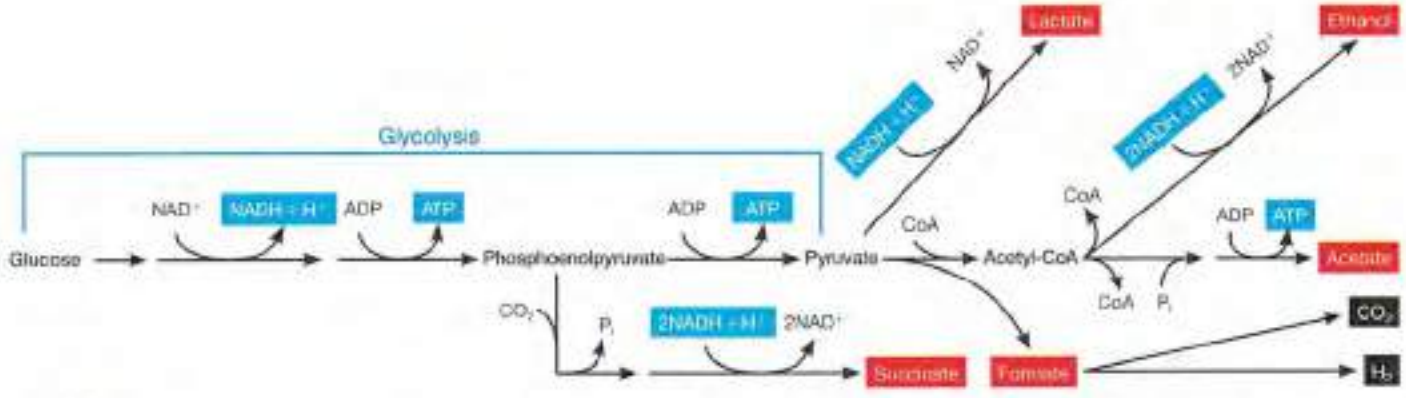


FIGURE **28.1 Mixed Acid Fermentation Pathway of *E. coli*** • *E. coli* is a representative methyl red positive organism and is recommended as a positive control for the test. Its mixed acid fermentation products (in order of abundance) are: lactate, carbon dioxide (CO_2), hydrogen gas (H_2), ethanol, acetate, succinate, and formate. Most of the formate is converted to H_2 and CO_2 gases. With the exception of succinate, all the end products are derived from pyruvate; succinate is produced from phosphoenolpyruvate. *Salmonella* and *Shigella* also are methyl red positive, though specific end-product profiles can differ.

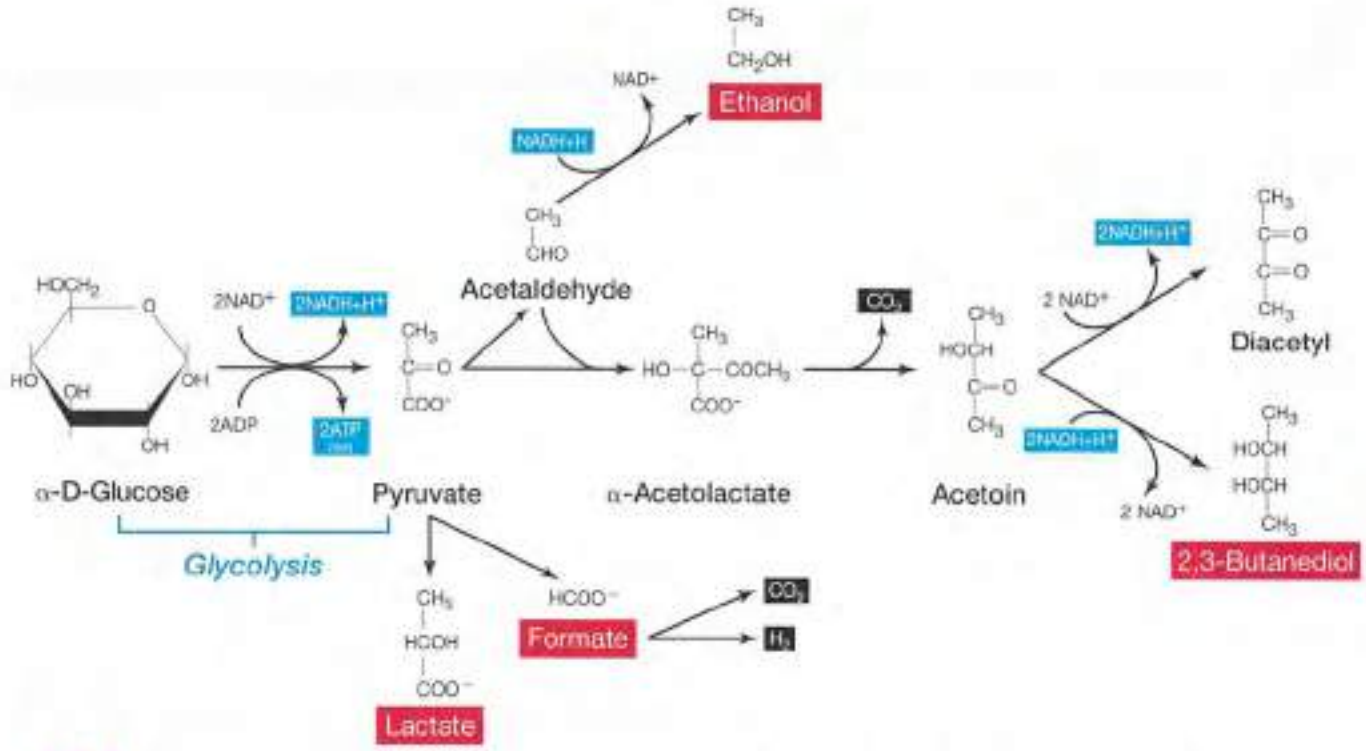


FIGURE **28.2** **Butanediol Fermentation Pathway** • Butanediol fermentation primarily produces the neutral molecules 2,3-butanediol and ethanol.

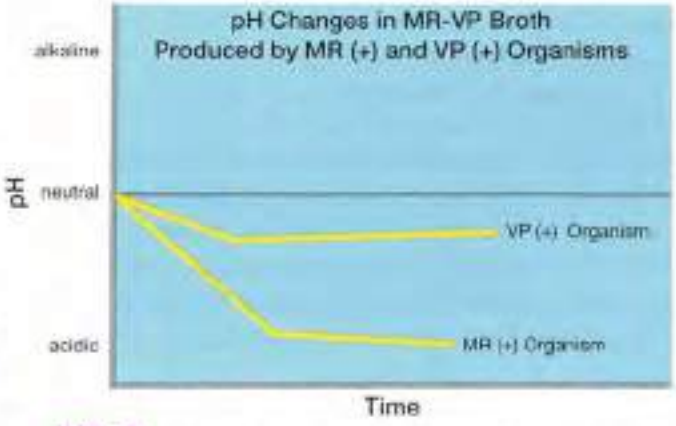


FIGURE **28.3** **Mixed Acid Versus Butanediol Fermentation and the Effects on MR-VP Media pH** • The MR-VP test differentiates between organisms that perform mixed acid fermentation and those that conduct butanediol fermentation. Mixed acid fermentation lowers the test broth's pH due to the abundance of acidic end products that are made. In contrast, organisms that perform butanediol fermentation make primarily neutral end products.

MR-VP Media Formulation

MR-VP broth is a defined media containing only peptone, glucose, and a phosphate buffer. The peptone provides protein, which is important as a nitrogen source. Glucose serves as the fermentable carbohydrate. The potassium phosphate buffer stabilizes the media's pH enough to guard against a significant pH decrease unless a substantive amount of acidic end products is made.

Interpreting MR-VP Results

For this test we inoculate only one tube of media, but after incubation we transfer 1 mL of the culture to a clean test tube to serve as the MR portion of the test and another 1 mL of culture is transferred to a tube and analyzed for the VP part of the test (Fig. 28.4).

Methyl Red (MR) Test

The MR portion of the test detects if mixed acid fermentation occurred. To assess if acidic end products are present or not, the pH indicator methyl red is added to 1 mL of the culture following incubation. Methyl red appears red at pH 4.4 and yellow at pH 6.2. Between these two pH values, it is various shades of orange. The development of a red color is the only true indication of a positive result (Fig. 28.5). As indicated in Table 28.1, a positive test result (red) means the bacterium performs mixed acid fermentation and converted glucose to acidic end products. A negative test result indicates that acidic end products were *not* detected, the test bacterium did *not* perform mixed acid fermentation.

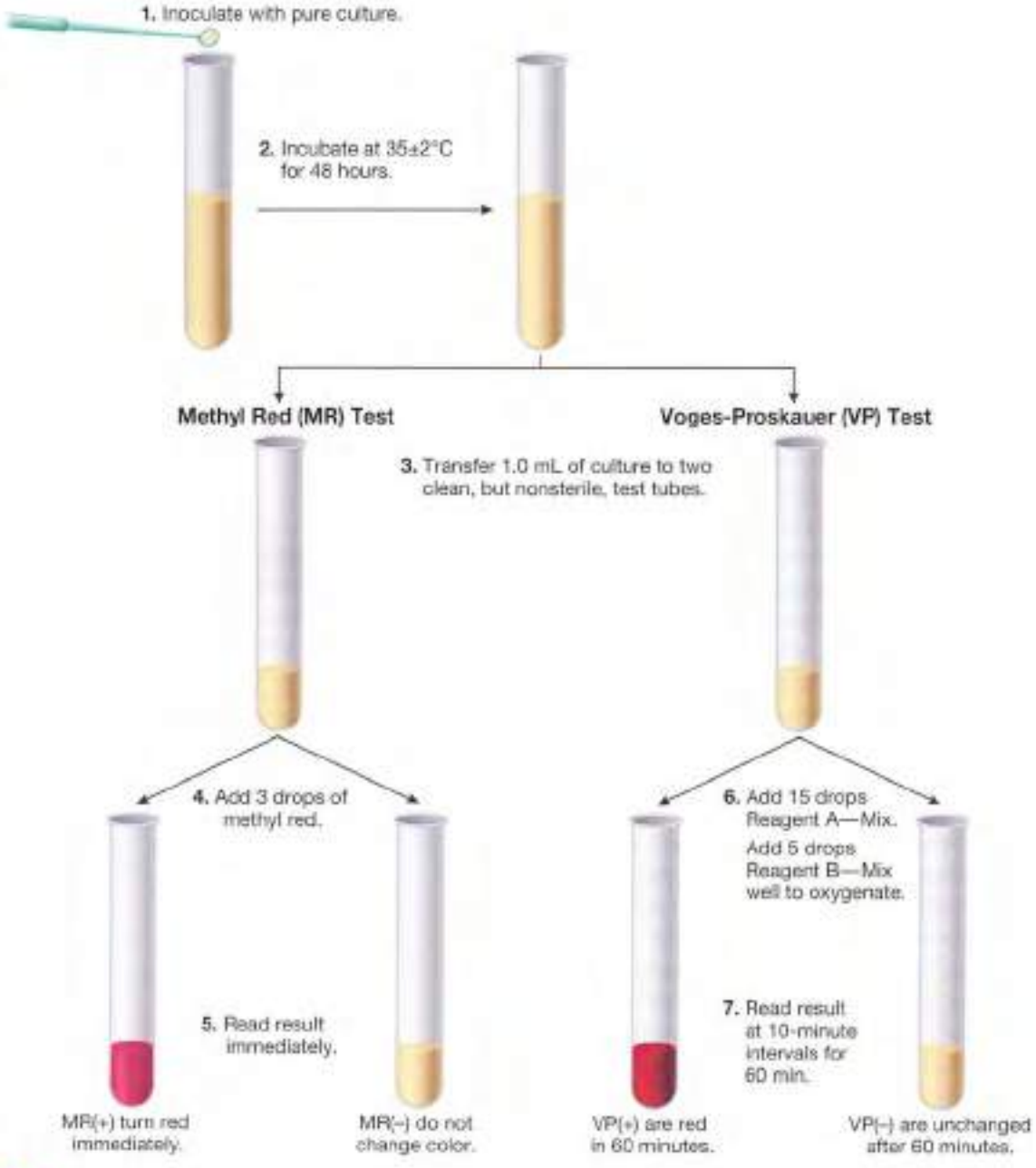

1. Inoculate with pure culture.
2. Incubate at 35±2°C for 48 hours.
Methyl Red (MR) Test
Voges-Proskauer (VP) Test
3. Transfer 1.0 mL of culture to two clean, but nonsterile, test tubes.
4. Add 3 drops of methyl red.
5. Read result immediately.
6. Add 15 drops Reagent A—Mix.
Add 5 drops Reagent B—Mix well to oxygenate.
7. Read result at 10-minute intervals for 60 min.
MR(+) turn red immediately.
MR(–) do not change color.
VP(+) are red in 60 minutes.
VP(–) are unchanged after 60 minutes.

FIGURE **28.4** **Procedural Diagram for the Methyl Red and Voges-Proskauer Tests** •

TABLE **28.1** **Methyl Red Test Results and Interpretations** •

Result	Interpretation	Symbol
Red	Organism performs a mixed acid fermentation	+
No color change	Organism does not perform a mixed acid fermentation (acidic end products were not made in detectable amounts)	–

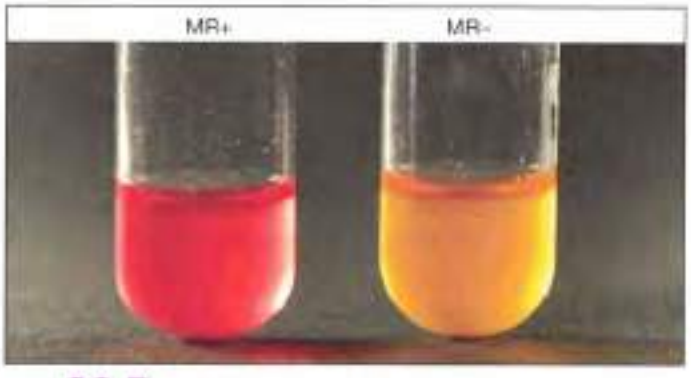


FIGURE **28.5** **Methyl Red Test Results** • A positive MR result appears red and means the tested bacterium performs mixed acid fermentation. A negative MR result is yellow and indicates the tested bacterium does not conduct mixed acid fermentation.

Voges-Proskauer (VP) Test

The Voges-Proskauer test reveals if a bacterium metabolized glucose via the butanediol fermentation pathway. As previously reviewed, butanediol fermentation mainly produces neutral end products (ethanol and 2,3-butanediol). Therefore, unlike the MR portion of the test, we can't simply check for an acidic pH to determine if the bacterium performed butanediol fermentation. Instead, we add reagents to the sample to determine if **acetoin**, an intermediate made in the butanediol fermentation pathway, is present.

First, VP Reagent A—a 5% α-naphthol (alpha-naphthol) solution—is added to the sample. This reagent acts as a color intensifier that allows for enhanced test sensitivity. After mixing well to oxygenate the media, VP Reagent B—a 40% potassium hydroxide (KOH) solution—is added. Once the KOH is added to raise the sample's pH, the tube is again vigorously mixed to enhance the sample's oxygenation. The increased sample oxygenation combined with the alkaline pH promotes the oxidation of acetoin to form **diacetyl** (Fig. 28.6). The resulting diacetyl then reacts with **guanidine-containing compounds** present in the media's peptone, to form a red color (Fig. 28.7).

As reviewed in Table 28.2, a positive VP result indicates the bacterium made acetoin; it metabolizes glucose via the butanediol fermentation pathway. Absence of a color change (or development of copper color) after adding the reagents indicates a negative test result, meaning the bacterium does *not* metabolize glucose via the butanediol fermentation pathway. The copper color is a result of interactions between the reagents and should not be confused with the true red color of a positive result. It's helpful to include positive and negative control for color comparison for this test.

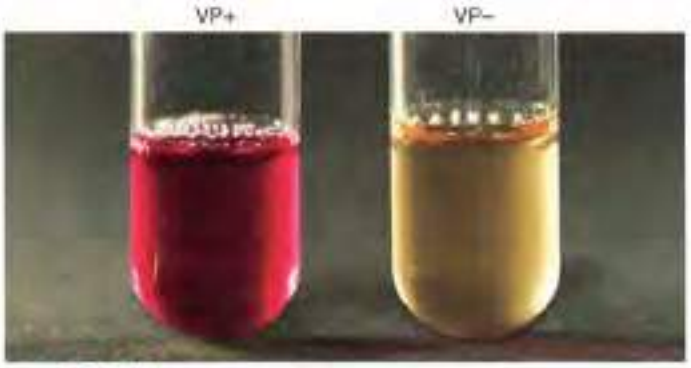


FIGURE 28.7 Voges-Proskauer Test Results • A positive VP test appears red and indicates acetoin was detected, thus butanediol fermentation occurred. A negative VP test appears yellow and indicates acetoin is not present, thus butanediol fermentation did not occur.

TABLE 28.2 Voges-Proskauer Test Results and Interpretations •

Result	Interpretation	Symbol
Red	Organism performs a 2,3-butanediol fermentation (acetoin was produced)	+
No color change	Organism does not perform a 2,3-butanediol fermentation (acetoin was not produced or it is not detectable)	–

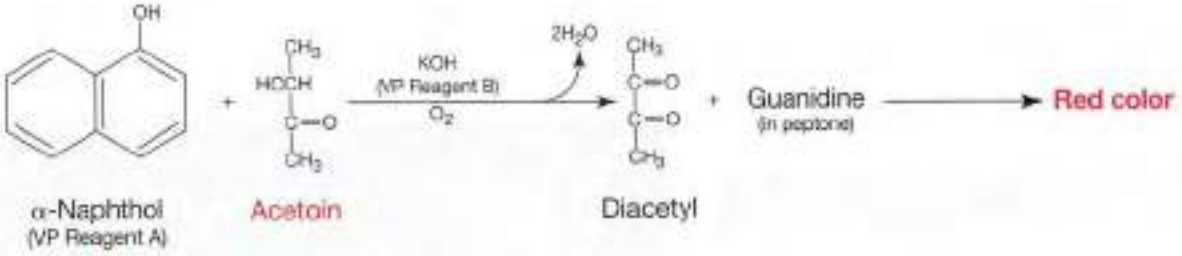


FIGURE 28.6 Voges-Proskauer Test Indicator Reaction • Adding potassium hydroxide (KOH) raises the sample's pH and promotes the oxidation of acetoin to form diacetyl. In this reaction, 5% α-naphthol enhances the development of the red color seen in a positive test result.

Clinical Applications

The methyl red and Voges-Proskauer tests are components of the *IMViC* battery of tests (*I*ndole, *M*ethyl red, *V*oges-Proskauer, and *C*itrate) used to distinguish between members of the family *Enterobacteriaceae* and differentiate them from other Gram-negative rods. Certain members of this family are clinically important because they cause diverse pathologies to include diarrheal illnesses, septicemia, pneumonia, meningitis, and urinary tract infections. Pathogens such as *Escherichia*, *Shigella*, and *Salmonella* are MR-positive and VP-negative. In contrast, certain other members of *Enterobacteriaceae*, such as *Enterobacter aerogenes* and *Klebsiella pneumoniae*, are MR-negative and VP-positive. Refer to Figure A.6 Sampling of Fermentation Pathways on page 473 to compare mixed acid and butanediol fermentation pathways to other fermentation pathways.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Methyl red and Voges-Proskauer (MR-VP) broths (3)
- ❑ Methyl red reagent
- ❑ VP Reagents A and B (formulations provided below)
- ❑ Nonsterile 12 mm × 75 mm test tubes (6)
- ❑ Nonsterile 1 mL pipettes (3)
- ❑ Fresh agar slant cultures of these recommended organisms:
  - ☐ *Escherichia coli*
  - ☐ *Enterobacter aerogenes*

Medium and Reagent Recipes

MR-VP Broth

Buffered peptone	7.0 g
Dipotassium phosphate (K_2HPO_4)	5.0 g
Dextrose (glucose) ($C_6H_{12}O_6$)	5.0 g
Distilled or deionized water	1.0 L

Adjust to pH 6.7–7.1 at 25°C

Methyl Red Reagent

Methyl red dye	0.1 g
Ethanol	300.0 mL
Distilled water to bring volume to	500.0 mL

VP Reagent A (5% α-naphthol solution)

α-naphthol	5.0 g
Absolute alcohol to bring volume to	100.0 mL

VP Reagent B (40% KOH)

Potassium hydroxide (KOH)	40.0 g
Distilled water to bring volume to	100.0 mL

PROCEDURE

Today you will perform methyl red and Voges-Proskauer tests on *Escherichia coli* and *Enterobacter aerogenes*. After a 48-hour incubation period, you will observe the results of a mixed acid fermentation and a 2,3-butanediol fermentation. The exercise involves transfer of a portion of broth containing living organisms and the addition of measured reagents to complete the observable reactions. Please use care in your transfers and in your measurements. Also, wear a lab coat, gloves, and chemical eye protection when performing this laboratory exercise. For help with the procedure, refer to Figure 28.4. You'll also find Tables 28.1 and Table 28.2 helpful.

1. **Obtain and Label Your Media:** Obtain three tubes of MR-VP media. Label two of them with the names of the organisms, your name, and the date. The third tube, in addition to being marked with your name and the date, should be labeled "control."
2. **Inoculate the Media:** Use an inoculating loop to inoculate your two test broths with the test cultures. Do not inoculate the control.
3. **Incubate the Samples:** Incubate your samples at 35±2°C for 48 hours.
4. **Clean up:** Dispose of stock cultures as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

→ Next Lab Session

1. **Obtain Your Samples:** Collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Mix and Transfer Your Samples:** Mix each broth well without splashing into the cap. Transfer 1.0 mL from each tube into two nonsterile tubes (Fig. 28.4). One tube will be designated for the MR test; the other will be for the VP test. Therefore, by the end of this step you should have one MR tube and one VP tube for each original sample (six tubes total if you started with three samples).

3 Develop Each Test and Make Observations:

Each MR test is processed as follows:

- Add 3 drops of methyl red reagent. Immediately observe the sample for the development of a red color.
- Using Table 28.1 as a guide, record your results in the table provided on the data sheet at the end of this exercise.

Each VP test is processed as follows:

- Add 15 drops (0.6 mL) of VP Reagent A. Mix well to oxygenate the medium, but be careful not to splash it.
- Add 5 drops (0.2 mL) of VP Reagent B. Carefully mix well again to oxygenate the medium.
- Place the tubes in a test-tube rack and observe for red color formation at regular intervals starting at 10 minutes for up to 1 hour. Mix again periodically to oxygenate the medium.
- Use Table 28.2 to help you interpret your results. Record your results in the table provided at the end of this exercise. (***Note:*** It is difficult to differentiate weak positive reactions from negative reactions. VP-negative reactions often produce a copper color. Weak VP-positive reactions produce a pink color. The more intense color change is usually observed at the media's surface.)

4 **Clean up:** Properly dispose of your samples as your instructor directs.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 28
DATA SHEET

Methyl Red and Voges-Proskauer (MR-VP) Tests

During the Lab

1 Observe your tubes and use Tables 28.1 and 28.2 to write up your results in the following table.

Organism	MR Result	VP Result	Interpretation
Uninoculated control			

Lab Related Questions

1 Select the true statement(s) about an MR-negative bacterium (select all that apply).

a. It performed mixed acid fermentation.
b. It converted glucose to acidic products.
c. It may convert glucose to acetoin.
d. It produced methyl red.
e. It may perform 2,3-butanediol fermentation.

2 Select the false statement(s) about a VP-positive bacterium (select all that apply).

a. It performs 2,3-butanediol fermentation.
b. It performs mixed acid fermentation.
c. It produces acetoin.
d. It converts glucose to mostly acidic end products.
e. It cannot metabolize glucose.

3 Choose the true statement(s) about fermentation (select all that apply).

a. Fermentation requires oxygen to occur.
b. All fermentation reactions start with glucose.
c. Fermentation reactions can occur in the presence of oxygen.
d. Glycolysis is the first stage of fermentation.
e. Pathogens do not conduct fermentation.

4 In this procedure, the samples were not transferred into sterile tubes for developing the MR or VP test. Why weren't sterile tubes required?

5 Cross out the steps that do not apply to the VP test, and then number the remaining steps so they are in the proper order.

_____ Add methyl red.

_____ Immediately observe the sample for a color change.

_____ Add 1 mL of your sample to a clean tube.

_____ Inoculate MR-VP broth media.

_____ Add α-naphthol.

_____ Add potassium hydroxide.

_____ Record a yellow result as having a positive VP test result.

6 Cross out the steps that do not apply to the MR test, and then number the remaining steps so they are in the proper order.

_____ Inoculate MR-VP broth media.

_____ Add 1 mL of your sample to a clean tube.

_____ Add potassium hydroxide.

_____ Add α-naphthol.

_____ Incubate sample for an hour.

_____ Record a positive MR test result if a red color develops.

_____ Add methyl red.

7 Which of the following occurs in 2,3-butanediol fermentation? (Select all that apply.)

a. Acetoin is made as an intermediate.
b. Hydrogen sulfide (H_2S) is made.
c. Glucose is metabolized.
d. Lactate is made.
e. The media's pH becomes alkaline.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 28
DATA SHEET
(continued)

Critical Thinking

1 Why is the methyl red test read immediately after addition of methyl red reagent, whereas the Voges-Proskauer test is read up to 60 minutes after addition of Reagents A and B?

2 In the VP test, the most intense color change is often at the media's surface. Based on the reaction's chemistry, why does this make sense?

— Lab Notes —

EXERCISE 29

Catalase Test

After doing this exercise, you should be able to:

1. Describe how the catalase test is performed.
2. Explain what the catalase test reveals about a bacterial species.
3. State the applications of the catalase test.
4. Perform a catalase test and interpret the test results.

Background

Organisms that perform aerobic respiration tend to generate toxic oxygen intermediates in the process. Unless such intermediates are broken down, they will damage cellular components. Common examples of toxic oxygen intermediates are hydrogen peroxide (H_2O_2) and superoxide radicals (O_2^-). These agents damage cells by oxidizing proteins and other biomolecules, thereby rendering them nonfunctional.

As a protective mechanism, organisms that perform aerobic respiration tend to make enzymes that break down toxic oxygen intermediates. For example, the enzyme **superoxide dismutase** catalyzes the conversion of superoxide radicals, which are particularly damaging toxic oxygen intermediates, to the comparatively less damaging molecule, hydrogen peroxide (Fig. 29.1). **Catalase,** an enzyme in aerobes, microaerophiles, and facultative anaerobes, converts hydrogen peroxide to water and gaseous oxygen (Fig. 29.2). In large part (though exceptions exist), the ability to make these protective enzymes accounts for an organism's ability to live in aerobic environments (Table 29.1). Oxygen is toxic to obligate anaerobes because these microbes lack enzymes such as catalase and superoxide dismutase to effectively break down toxic oxygen intermediates that tend to evolve under aerobic conditions.

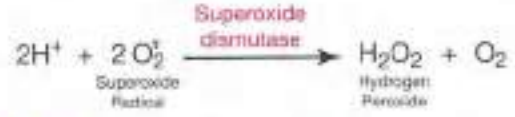


FIGURE 29.1 Microbial Production of H_2O_2 • Superoxide dismutase produces hydrogen peroxide in the process of detoxifying superoxide radicals (O_2^-) made in aerobic cellular respiration.

FIGURE 29.2 Catalase Breaks Down H_2O_2 • Catalase converts hydrogen peroxide to water and oxygen gas.

TABLE 29.1 Protective Enzymes in Various Aerotolerance Groups •

Aerotolerance Group	Superoxide Dismutase	Catalase
Obligate aerobe	Present	Present
Facultative anaerobe	Present	Present
Microaerophile	Present	Present in small amounts
Aerotolerant anaerobe	Present	Absent (alternative mechanism)
Obligate anaerobe	Absent	Absent

Interpreting Catalase Test Results

By exposing bacteria to a 3% hydrogen peroxide solution (typical store-grade hydrogen peroxide), we can determine whether they make catalase or not. Upon adding hydrogen peroxide to a catalase-positive culture, oxygen gas bubbles immediately form. If bubbles do not form, the organism is catalase-negative (Table 29.2).

TABLE 29.2 Catalase Test Results and Interpretations •

Result	Interpretation	Symbol
Bubbles immediately develop (immediate effervescence)	Catalase is present; the organism is aerobic	+
No bubbles detected	Catalase is absent or not detectable	–

This test is often performed by adding hydrogen peroxide (Fig. 29.3) to a bacterial smear on a microscope slide. If using this "slide method," you should immediately cover the sample after adding hydrogen peroxide. This reduces workspace contamination from the aerosols a positive test may generate as bubbles develop (Fig. 29.4).

Another way to perform the catalase test is to add hydrogen peroxide directly to bacteria growing on an agar slant (Fig. 29.5) or on an agar plate. *Note:* Do not add hydrogen peroxide to cultures growing on blood agar because a false-positive result is likely to develop as catalase in the red blood cells in the agar breaks down the hydrogen peroxide. Lastly, upon adding hydrogen peroxide to the agar's surface (be it a slant or a plate culture), the culture will not be usable for other tests. As such, before performing the test in this way, ensure you do not need the culture for other applications.

A Note on Handling Hydrogen Peroxide

Hydrogen peroxide is a colorless liquid with disinfectant and bleaching properties. It is an irritant, so you should wear safety glasses when handling this substance. Also, this molecule is naturally unstable and decomposes slowly to form water and oxygen gas. Ultraviolet light and heat increase the rate of H_2O_2 decomposition, which is why we store the solution in a dark bottle in a cool place.

FIGURE **29.3** **Catalase Slide Test Results** • Visible bubbles upon adding hydrogen peroxide to a bacterial smear indicates a positive result in the catalase slide test. A catalase-negative organism is on the left. A catalase-positive organism is on the right. (For photography purposes, the slide was not covered, but you should cover the sample as shown in Fig. 29.4.)

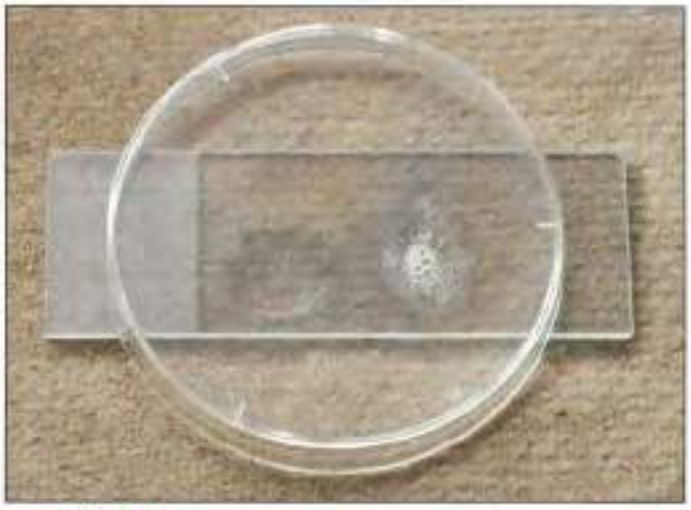


FIGURE **29.4** **Cover the Reaction** • Upon adding the hydrogen peroxide to the slide, cover it with a petri plate lid or base to prevent spattering from a positive result. When the reaction has subsided, the lid can be removed, disinfected, and saved for subsequent tests.

FIGURE **29.5** **Catalase Agar Slant Test Results** • The catalase test may also be performed by adding hydrogen peroxide to bacteria growing on an agar slant. A catalase-positive organism is on the left. A catalase-negative organism is on the right. In a similar fashion, you can also use a culture growing on an agar plate.

Clinical Applications

This test is useful for differentiating Gram-positive bacteria that make catalase from Gram-positive bacteria that do not. It's especially useful for the differentiation of catalase-positive *Staphylococcus* from catalase-negative *Streptococcus*. It can also differentiate catalase-positive *Micrococcus* species from the catalase-negative *Enterococcus*, and *Lactococcus* species. Variations on this test also help presumptively identify *Mycobacterium*, a genus that includes the causative agents of tuberculosis and leprosy.

Not only do catalase-positive bacteria typically thrive in an aerobic environment, but a high level of catalase is associated with pathogenicity (ability to cause disease). For example, some bacteria use catalase to thwart immune defenses by breaking down the hydrogen peroxide that certain phagocytic white blood cells (e.g., macrophages) use to kill bacteria.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ H_2O_2 (hydrogen peroxide, fresh 3% solution in amber dropper bottles)
- ❑ Microscope slides
- ❑ Petri plate base or lid
- ❑ Sterile wooden applicator or sterile cotton swab
- ❑ Nutrient agar (NA) slant (1, as an uninoculated control)
- ❑ Fresh (18- to 24-hour) agar slant cultures of these recommended organisms:
  - ☐ *Staphylococcus epidermidis*
  - ☐ *Lactococcus lactis*

PROCEDURE

For this exercise, you will perform the catalase test. Follow your instructor's directions regarding which version(s) of the test you will perform. They may have you perform the slide version and/or the slant version of the test. If you perform *both* versions, then you must perform the slide test first. This is because the slant version requires adding H_2O_2 directly to the slant, after which the culture is not usable. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

Slide Test

1. **Obtain Your Samples:** Obtain the test culture(s) from your instructor.
2. **Transfer Bacteria to a Slide:** Use a sterile wooden applicator or sterile cotton-tipped swab to smear a visible amount of bacteria onto a microscope slide. Properly dispose of the transfer tool. ***Note***: You must use fresh (18- to 24-hour) cultures for this test because older cultures may not have enough active catalase to produce visible bubbles.
3. **Add Hydrogen Peroxide to the Sample:** Aseptically place 1 or 2 drops of H_2O_2 directly onto the bacteria and immediately cover the sample with the base or lid of a petri plate (Fig. 29.4). This will contain any spattering due to bubbling that develops for a catalase-positive result. Observe the sample to see if bubbles develop. You can remove the cover if bubbling is absent or has subsided. ***Note***: When running this test on an unknown, you should simultaneously test a positive control to verify the quality of the peroxide.
4. **Record Your Data:** Record your results in the table at the end of the lab exercise.
5. **Clean up:** Dispose of the materials as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

Agar Slant Test

1. **Obtain Your Samples:** Obtain the test culture(s) from your instructor.
2. **Add Hydrogen Peroxide to the Sample:** Before performing this test, confirm that you do not need the culture(s) for any other applications. Next, add approximately 1 mL of H_2O_2 to each of the three slants (two cultures and an uninoculated control) and replace the caps. Process the slants *one at a time*, observing and recording the results as you go. Refer to Table 29.2 to help you interpret the results.
3. **Record Your Data:** Record your results in the table at the end of the lab exercise.
4. **Clean up:** Dispose of the materials as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 29

DATA SHEET

Catalase Test

During the Lab

1 Observe your samples and use Table 29.2 to write up your results in the following table.

Organism	Bubbles? Y/N	+/–	Interpretation

Lab Related Questions

1 Oxygen is toxic to obligate anaerobes because these bacteria:

a. Make superoxide dismutase, which generates H_2O_2 from oxygen radicals.

b. Produce catalase, which produces H_2O_2 in aerobic environments.

c. Conduct anaerobic respiration, which produces oxygen radicals.

d. Cannot detoxify toxic oxygen intermediates.

e. Make catalase to breakdown H_2O_2.

2 Which of the following bacterial groups would you expect to have superoxide dismutase and catalase? (Select all that apply.)

a. Obligate aerobes

b. Obligate anaerobes

c. Facultative anaerobes

d. Aerotolerant anaerobe

e. Microaerophiles

3 Assume you grow a bacterium on blood agar and then perform the catalase test by adding H_2O_2 directly to the bacteria growing on the agar surface. Upon adding the peroxide, you see bubbles. Based on this, what could you conclude?

a. The organism is catalase positive.

b. The organism can break down H_2O_2.

c. The organism is an obligate aerobe.

d. The organism is an obligate anaerobe.

e. You cannot make a reliable conclusion as this may be a false-positive result.

4 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ Hydrogen peroxide is naturally stable.

_____ Certain phagocytic white blood cells use H_2O_2 as a defense mechanism against bacteria.

_____ Obligate aerobes cannot effectively detoxify toxic oxygen intermediates.

_____ Hydrogen peroxide degrades to water and hydrogen gas.

5 If an organism does not have catalase then:

a. It must be a prokaryotic cell.
b. It is less able to tolerate aerobic environments.
c. It cannot break down superoxide radicals.
d. It cannot metabolize nutrients.
e. It is not a pathogen.

Critical Thinking

1 In some cases the inclusion of an uninoculated control is recommended. In such a case, would the uninoculated control be a positive or a negative control? Explain your reasoning.

2 A negative catalase test is not indicative of a lack of aerobic respiration. Explain why.

EXERCISE 30

Oxidase Test

After doing this exercise, you should be able to:

1. Explain what the oxidase test reveals about a bacterial species.
2. State what cytochrome c oxidase is, where it's found, and what it does.
3. Describe how the oxidase test is performed.
4. State the applications of the oxidase test.
5. Perform an oxidase test and interpret the test results.

Background

The oxidase test reveals if a bacterium has **cytochrome c oxidase**, a participant in certain aerobic electron transport chains. Recall that electron transport chains facilitate the last stage of cellular respiration where oxidative phosphorylation generates ATP.

In ETCs, electrons pass from one membrane-associated electron carrier to the next until they reach a final electron acceptor—oxygen in aerobic respiration or something other than oxygen, such as nitrate or sulfate, in anaerobic respiration. The types of electron carriers that participate in ETCs are diverse (Fig. 30.1). In many cases the ETCs in prokaryotic cells resemble the ETCs in the mitochondria of eukaryotic cells. Furthermore, some organisms have more than one type of ETC and may alternate which ETCs they

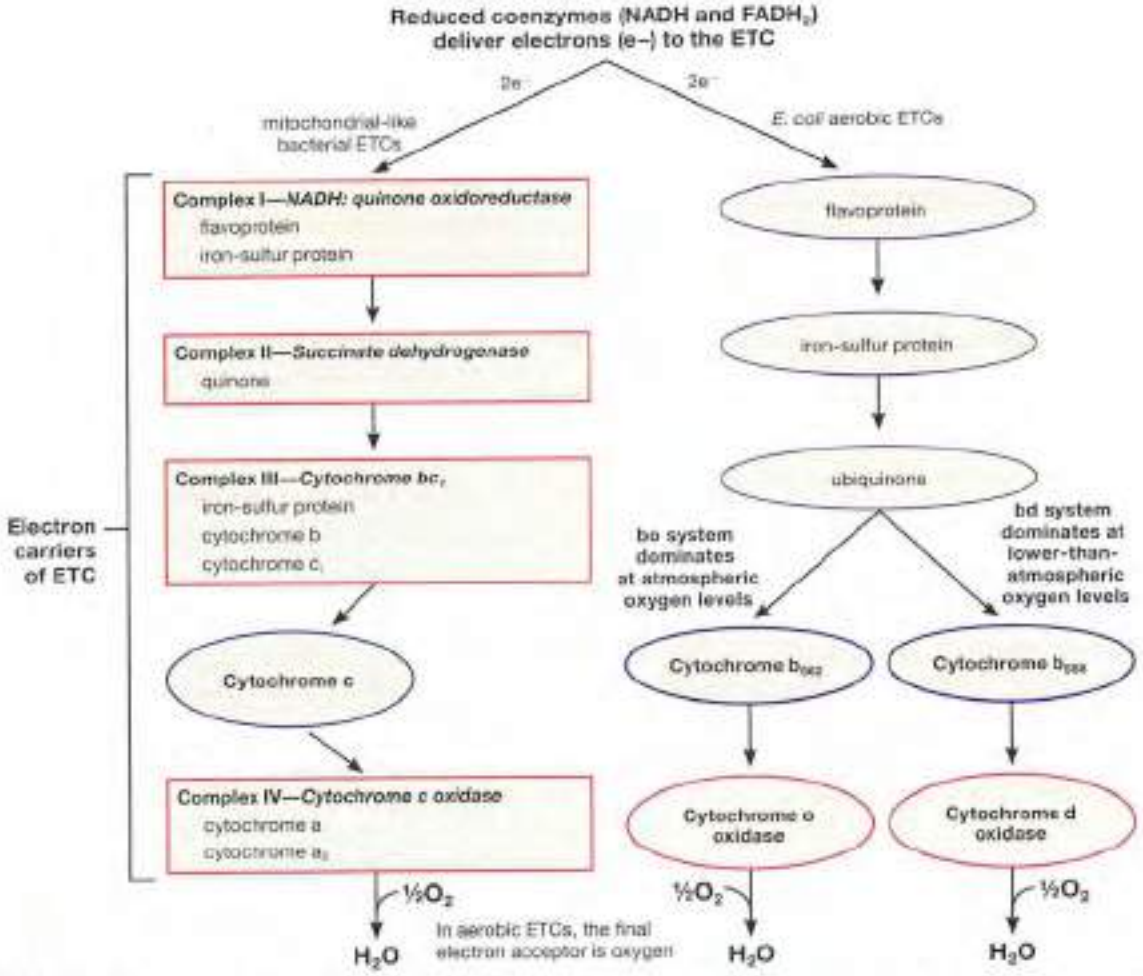


FIGURE **30.1 Aerobic Electron Transport Chains (ETCs)** • (**Left**) Bacteria with chains resembling the mitochondrial ETC in eukaryotes contain cytochrome c oxidase (Complex IV), which transfers electrons from cytochrome c to oxygen. (**Middle** and **Right**) In the aerobic ETCs of *E. coli*, oxygen doesn't bind to the bo system (**Middle**) as well as it binds to the bd system (**Right**). Consequently, the bd system predominates under decreased oxygen levels. Therefore, the bd system allows *E. coli* to continue using aerobic respiration even when oxygen levels are low. Under low oxygen conditions, microbes without the bd system must either resort to less efficient oxygen independent ATP production pathways (anaerobic respiration or fermentation) or forgo ATP production and die.

use, depending on the availability of oxygen or other final electron acceptor(s). For example, *Escherichia coli* has two different types of aerobic ETCs as well as at least one type of anaerobic ETC that requires nitrate as the final electron acceptor.

While dissecting the diverse components of ETCs is an informative practice, for the purposes of this laboratory exercise we are primarily interested in only one electron carrier, cytochrome c oxidase, which is involved in some (though not all) aerobic respiratory chains. This carrier transfers electrons from cytochrome c to oxygen, the final electron acceptor in aerobic respiration.

How the Oxidase Test Works

The chemistry of ETCs is essentially a collection of redox (reduction-oxidation) reactions. As a reminder, in reduction reactions a molecule or atom gains one or more electrons; whereas, in an oxidation reaction, an atom or molecule loses one or more electrons. In nature, cytochrome c oxidase does exactly as its name implies, it oxidizes the electron carrier cytochrome c. The electron that cytochrome c oxidase obtains is then delivered to oxygen, thereby producing water (Fig. 30.2).

In the oxidase test, we establish a scenario in which cytochrome c oxidase obtains electrons from an added reagent instead of oxidizing its natural electron donor, cytochrome c. The reaction depends on adding a **chromogenic reducing agent** (color-generating agent) called **tetramethyl-*p*-phenylenediamine (TMPD)**, to serve as an electron donor. Upon oxidation, this chromogenic reducing agent develops a purple/dark-blue color. In the reaction, if cytochrome c oxidase is present, then it oxidizes TMPD thereby generating a purple/blue coloration (Fig. 30.3).

We can conclude that bacteria that have cytochrome c oxidase respire aerobically. However, a negative oxidase test result only means the bacterium lacks cytochrome c oxidase; it does not necessarily mean the bacteria cannot respire aerobically using some other ETC that doesn't require cytochrome c oxidase. For example, members of the *Enterobacteriaceae*, are capable of aerobic respiration, but they have a different terminal oxidase system and give a negative result for the oxidase test. There are several ways to perform the oxidase test. You may:

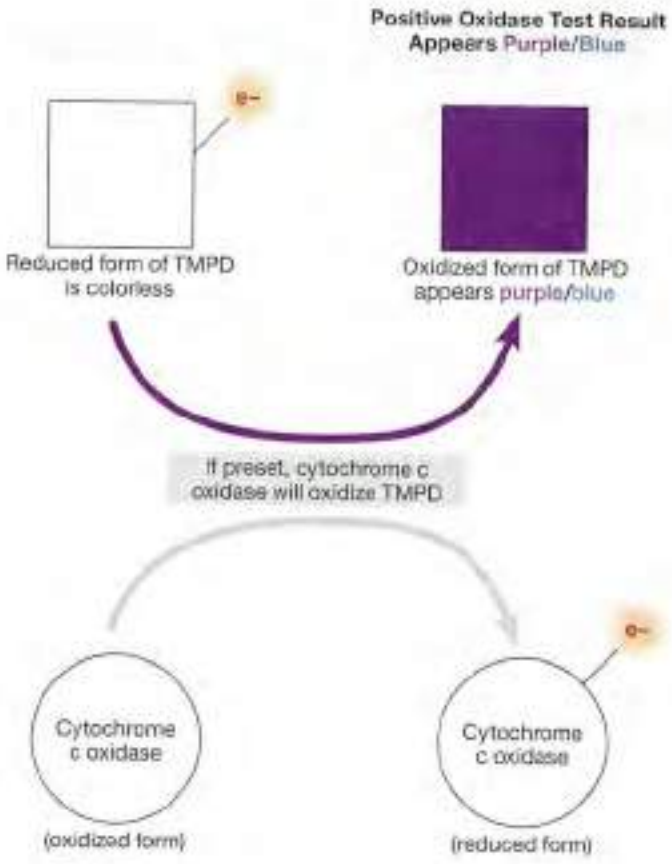


FIGURE 30.3 Oxidation of Tetramethyl-*p*-phenylenediamine (TMPD) by Cytochrome c Oxidase • If cytochrome c oxidase is present, it oxidizes TMPD. The oxidized form of TMPD appears purple/blue.

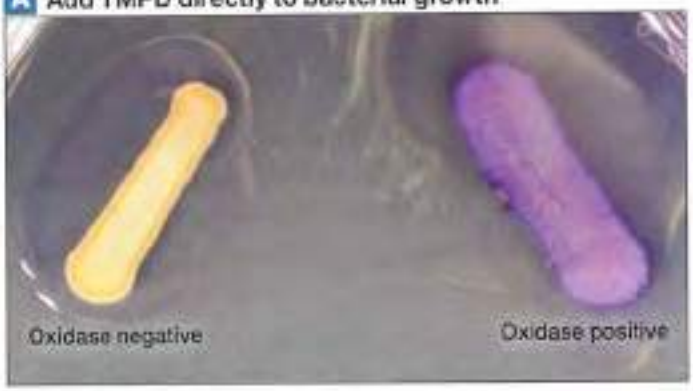
1. Add the TMPD reagent directly to bacterial growth on solid media (Fig. 30.4A); or
2. Smear the test bacterium onto filter paper, and then add TMPD to the smear (Fig. 30.4B); or
3. Smear the test bacterium onto paper infused with the TMPD reagent (Fig. 30.4C).

Regardless of the approach applied, if cytochrome c oxidase is present a color change to purple or blue occurs within seconds as TMPD is oxidized. Lack of a color change within the allotted time means that cytochrome c oxidase is not present and signifies a negative result (Table 30.1).

$$2\ \text{Cytochrome } c_{red} + 2H^+ + \tfrac{1}{2}O_2 \xrightarrow{\text{cytochrome c oxidase}} 2\ \text{Cytochrome } c_{ox} + H_2O$$

FIGURE 30.2 Natural Oxidation of Cytochrome c • Under natural conditions, cytochrome c oxidase removes electrons from cytochrome c (oxidizes cytochrome c) and transfers those electrons to O_2, producing water.

A Add TMPD directly to bacterial growth



B Smear bacteria on filter paper and then add TMPD

C Smear bacteria onto TMPD-infused paper

FIGURE **30.4** Three Ways to Perform the Oxidase Test •

TABLE **30.1** Oxidase Test Results and Interpretations •

Result	Interpretation	Symbol
Dark blue/purple within 20 seconds	Cytochrome c oxidase is present; the organism can perform aerobic respiration.	+
No color change to blue/purple within 20 seconds	Cytochrome c oxidase is not present or not detectable (***Note:*** Do not assume the tested bacterium is anaerobic. The bacterium may have alternative aerobic ETCs that do not require cytochrome c oxidase.)	–

Avoiding Inaccurate Results

False-positive oxidase test results can develop if TMPD autoxidizes; that is, TMPD is oxidized independent of cytochrome c oxidase action. The most common teaching lab scenarios that trigger false-positive results are exposing the TMPD to a nickel-base alloy wire inoculating loop and/or excessive exposure of TMPD to atmospheric oxygen. To avoid these scenarios, only use a wooden applicator or cotton-tipped swab to apply the bacteria to the test and make your observations immediately upon adding the TMPD reagent.

False-negative oxidase test results can occur if cytochrome c oxidase activity is inhibited. This can occur if the cultures are older/not actively growing and/or if acids are present (pH below 5.1). As such, to avoid false-negative results, use fresh cultures that are 18–24 hours old and avoid using cultures grown under conditions that favor fermentation (e.g., don't use bacteria grown on/in media that detect fermentation).

Clinical Applications

Cytochrome c oxidase is an essential participant in aerobic respiration in animals. When one considers that cyanide, azide, and carbon monoxide are all deadly because they inhibit cytochrome c oxidase, it's easy to appreciate just how important this enzyme is. Because ETCs are often evolutionarily conserved, we can find this enzyme in certain aerobic bacteria.

In clinical laboratories, this test is useful for identifying a number of oxidase-positive pathogens of the *Neisseria*, *Moraxella*, *Campylobacter*, and *Pasteurella* genera. For example, the oxidase test can help differentiate *N. gonorrhoeae* (Gram-negative/oxidase-positive bacteria that cause the sexually transmitted infection gonorrhea) from *Chlamydia trachomatis*, a Gram-negative/oxidase-negative bacterium that causes chlamydia (a sexually transmitted infection with similar signs and symptoms as gonorrhea). The oxidase test is also useful in differentiating the oxidase-negative *Enterobacteriaceae* from the oxidase-positive *Pseudomonadaceae*.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Sterile wooden applicator, sterile swab, or disposable plastic loop
- ❑ Small pieces of filter paper
- ❑ Sterile water
- ❑ Oxidase test reagent (TMPD solution)
- ❑ 24-hour agar slant cultures of these recommended organisms:
  - ☐ *Escherichia coli*
  - ☐ *Neisseria* species (e.g., *N. sicca* or *N. flava*)

Reagent Recipe

Oxidase Test Reagent

◗ Tetramethyl-*p*-phenylenediamine dihydrochloride	1.0 g
◗ Distilled or deionized water	100.0 mL

PROCEDURE

For this exercise, you will perform the oxidase test. Here we describe the oxidase test using filter paper and reagent. If you are using a commercial kit, follow its instructions. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

1. **Obtain Your Samples:** Obtain fresh test culture(s) from your instructor.
2. **Smear Bacteria onto Filter Paper:** Obtain a piece of filter paper and lay it on a paper towel. Then, use a sterile wooden applicator, sterile swab, or disposable loop to smear some of the culture onto the filter paper. Properly dispose of the applicator/loop.
3. **Add TMPD (Oxidase Reagent):** Add a few drops of TMPD (oxidase reagent) to the smear and observe for a color change within 20 seconds. *Note*: If running an unknown sample, you should also test a known oxidase-positive organism.
4. **Repeat the Test for Other Samples:** Repeat Steps 2 and 3 for additional test organisms. The same filter paper can be used if there is room. When finished, properly dispose of the filter paper into a biowaste container.
5. **Record Your Data:** Record your results in the table at the end of the lab exercise.
6. **Clean up:** Dispose of the materials as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 30

DATA SHEET

Oxidase Test

During the Lab

1 Using Table 30.1 as a guide to help analyze your results, record them in the following table.

Organism	Color Result	+/–	Interpretation

Lab Related Questions

1 In nature, cytochrome c oxidase normally reduces:

a. Water.
b. Oxygen.
c. Cytochrome c.
d. TMPD.
e. Carbon monoxide.

2 Which of the following would not have cytochrome c oxidase? (Select all that apply.)

a. Obligate aerobes
b. Obligate anaerobes
c. Facultative anaerobes
d. Aerotolerant anaerobe
e. Human cells

3 In nature, cytochrome c oxidase normally oxidizes:

a. Water.
b. Oxygen.
c. Cytochrome c.
d. TMPD.
e. Carbon monoxide.

4 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ TMPD serves as an oxidizing agent in the oxidase test.

_____ Bacteria that lack cytochrome c oxidase may be anaerobic.

_____ Bacteria that perform aerobic respiration always have cytochrome c oxidase.

_____ If TMPD autoxidizes, then you may observe a false-negative result.

5 If an organism has cytochrome c oxidase then:

a. It must be a prokaryotic cell.
b. It can conduct aerobic respiration.
c. It will reduce TMPD.
d. It will die if oxygen is absent.
e. It is not a pathogen.

6 The oxidase test can differentiate (select all that apply):

a. *Neisseria* species from one another.
b. *Neisseria* species from *Moraxella* species.
c. *Neisseria gonorrhoeae* from *Enterobacteriaceae* members.
d. *Neisseria gonorrhoeae* from *Chlamydia trachomatis*.
e. Members of *Enterobacteriaceae* from *Chlamydia trachomatis*.

Critical Thinking

1 Assume you obtain the following data for a collection of oxidase tests performed on various 20-hour cultures of Organism A.

Growth Media	Oxidase Test Results		
	Observed at 10 Seconds	Observed at 30 Seconds	Observed at 1 Minute
Nutrient Agar (NA)	–	+	+
Tryptic Soy Agar (TSA)	+	+	+
Eosin Methylene Blue (EMB)	–	–	–

Based on the data, would you conclude Organism A is oxidase positive or oxidase negative? Explain your reasoning. Your explanation should include a discussion of why the data are conflicting despite performing the tests on the same organism.

2 You are performing an oxidase test and notice the positive control is not generating a blue color when exposed to TMPD. However, your test sample is clearly oxidizing TMPD. Assuming you performed the procedure correctly and used the same bottle of TMPD for both samples, what is the most likely explanation for the discrepancy in the test results?

EXERCISE 31

Nitrate Reduction Test

After doing this exercise, you should be able to:

1. Explain what the nitrate reduction test reveals about a bacterial species.
2. Describe how to perform and interpret the nitrate reduction test.
3. State the applications of the nitrate reduction test.
4. Perform a nitrate reduction test and interpret the test results.

Background

The nitrate test is a qualitative procedure that allows us to differentiate bacterial species from one another based on their ability to reduce nitrate (NO_3^-). Nitrate is one example of a final electron acceptor in anaerobic respiratory chains.

Nitrate reduction is complex; organisms may perform nitrate reduction with diverse outcomes (Fig. 31.1). For example, members of the *Enterobacteriaceae* use an enzyme called **nitrate reductase** to perform a single-step reaction in which a reduction reaction reduces nitrate to nitrite (NO_2^-). Other bacteria, called "denitrifiers," perform a multistep process called **dissimilatory nitrate reduction** (sometimes also called denitrification). In this process, nitrate is reduced to form molecular nitrogen (N_2) or nitrous oxide (N_2O), both of which are gases. Denitrifiers are ecologically important in the nitrogen cycle. Other organisms perform **assimilatory nitrification**, a process in which nitrate or nitrite is reduced to form ammonium (NH_4^+), which may then be used to build amino acids.

Interpreting the Nitrate Reduction Test

Nitrate broth is a complex (or enriched) medium of beef extract, peptone, and potassium nitrate (KNO_3). An inverted Durham tube in each broth serves to trap a portion of any produced gas. Following incubation, we analyze the test via a three-step process (Fig. 31.2).

First, check if there is a bubble in the Durham tube. If there is a bubble *and* you are certain the bacterium does *not* conduct fermentation, then you may safely conclude the tested organism reduces nitrate to gaseous end products such as molecular nitrogen (N_2) or nitrous oxide (N_2O) (Fig. 31.3A). (For more information on fermentation, see Ex. 27: Phenol Red Fermentation Broth.)

Because fermentation pathways can generate gas byproducts such as CO_2, a bubble in the Durham tube is not a specific indictor of nitrate reduction. As such, in most cases you should process each sample by adding a variety of reagents. First, nitrate Reagent A (sulfanilic acid) and nitrate Reagent B (α-naphthylamine) are added to the medium to test for nitrate reduction to nitrite. If present, nitrite will form nitrous acid (HNO_2) in the aqueous medium (Fig. 31.4). Nitrous acid reacts with the added reagents to produce a red, water-soluble compound (Fig. 31.3B). So, if you see a red color after adding Reagents A and B, then you may conclude the tested bacterium reduces nitrate to nitrite.

However, if you do *not* see a color change to red after adding Reagents A and B, then you must add nitrate Reagent C (zinc dust) to determine if the bacterium reduced nitrate beyond nitrite. Zinc dust catalyzes the reduction of any nitrate still in the medium to nitrite. The generated nitrite spontaneously becomes nitrous

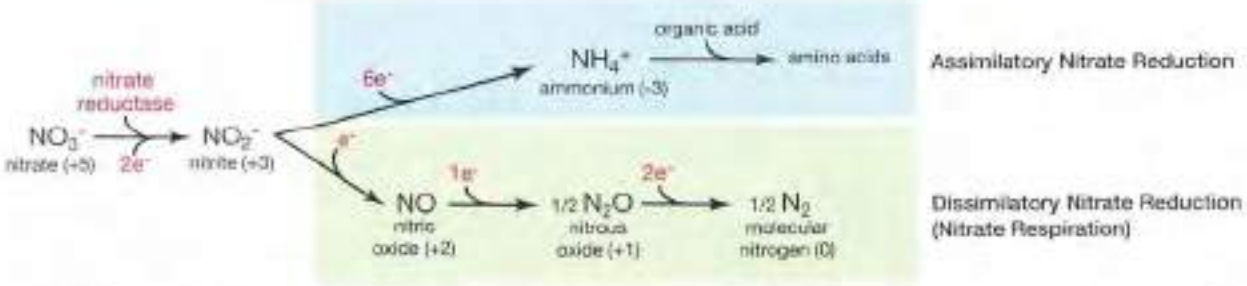


FIGURE **31.1** **Possible End Products of Nitrate Reduction** • In the presence of nitrate reductase, nitrate (NO_3^-) undergoes a single reduction to form nitrite (NO_2^-). Nitrite can then be further reduced via dissimilatory nitrate reduction or assimilatory nitrification. In each compound, nitrogen's oxidation state is shown in parentheses. Notice that as electrons are added, nitrogen's oxidation state decreases.

acid, which reacts with the already present Reagents A and B to turn the medium red. In this instance, the red color indicates the organism did *not* reduce nitrate (Fig. 31.3C). Therefore, lack of a color change after adding Reagent C indicates the organism reduced nitrate to NH_4^+, NO, N_2O, or some other nitrogenous compound other than nitrite.

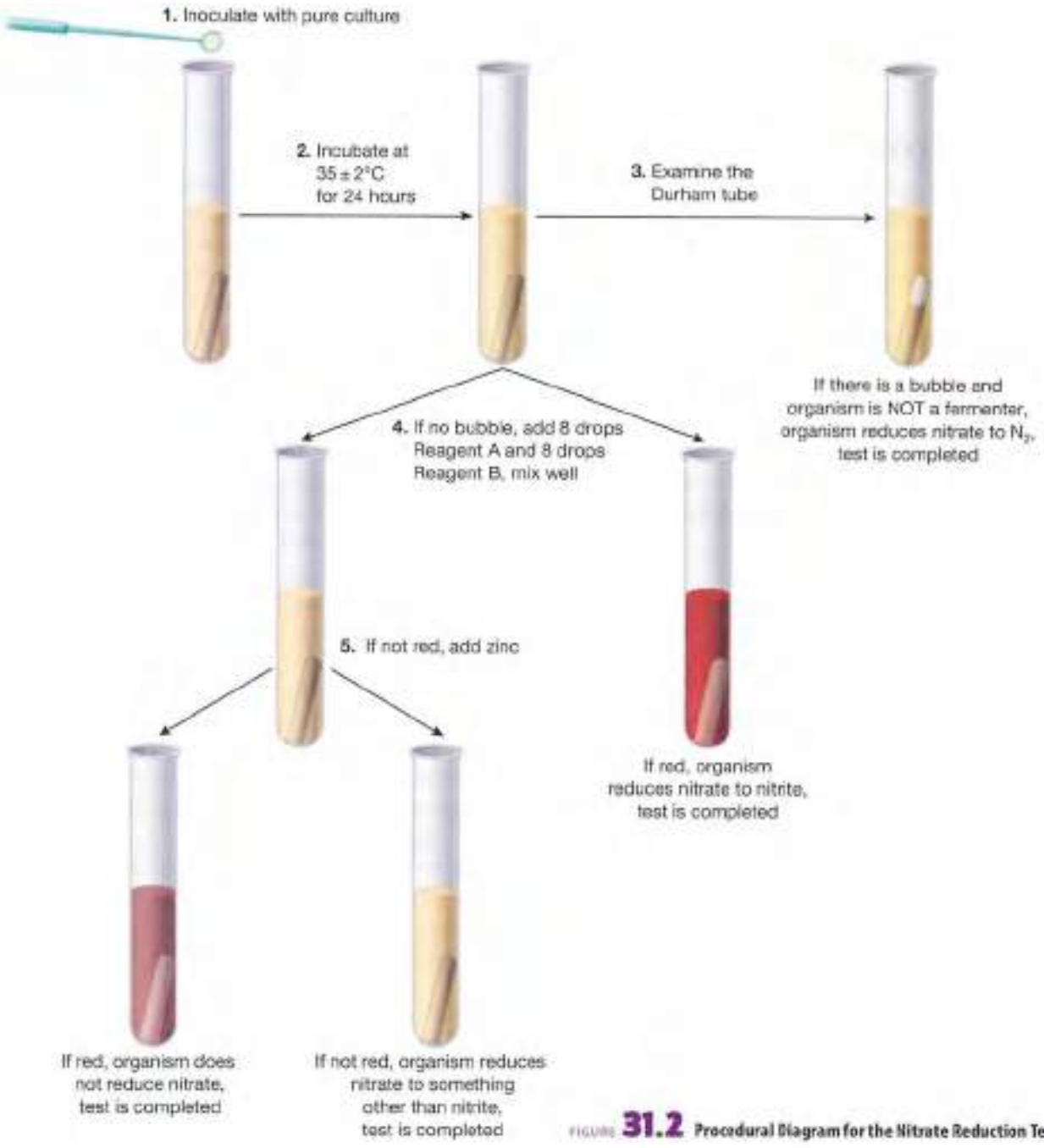

1. Inoculate with pure culture
2. Incubate at 35 ± 2°C for 24 hours
3. Examine the Durham tube
If there is a bubble and organism is NOT a fermenter, organism reduces nitrate to N_2, test is completed
4. If no bubble, add 8 drops Reagent A and 8 drops Reagent B, mix well
5. If not red, add zinc
If red, organism reduces nitrate to nitrite, test is completed
If red, organism does not reduce nitrate, test is completed
If not red, organism reduces nitrate to something other than nitrite, test is completed

FIGURE **31.2** **Procedural Diagram for the Nitrate Reduction Test** ●

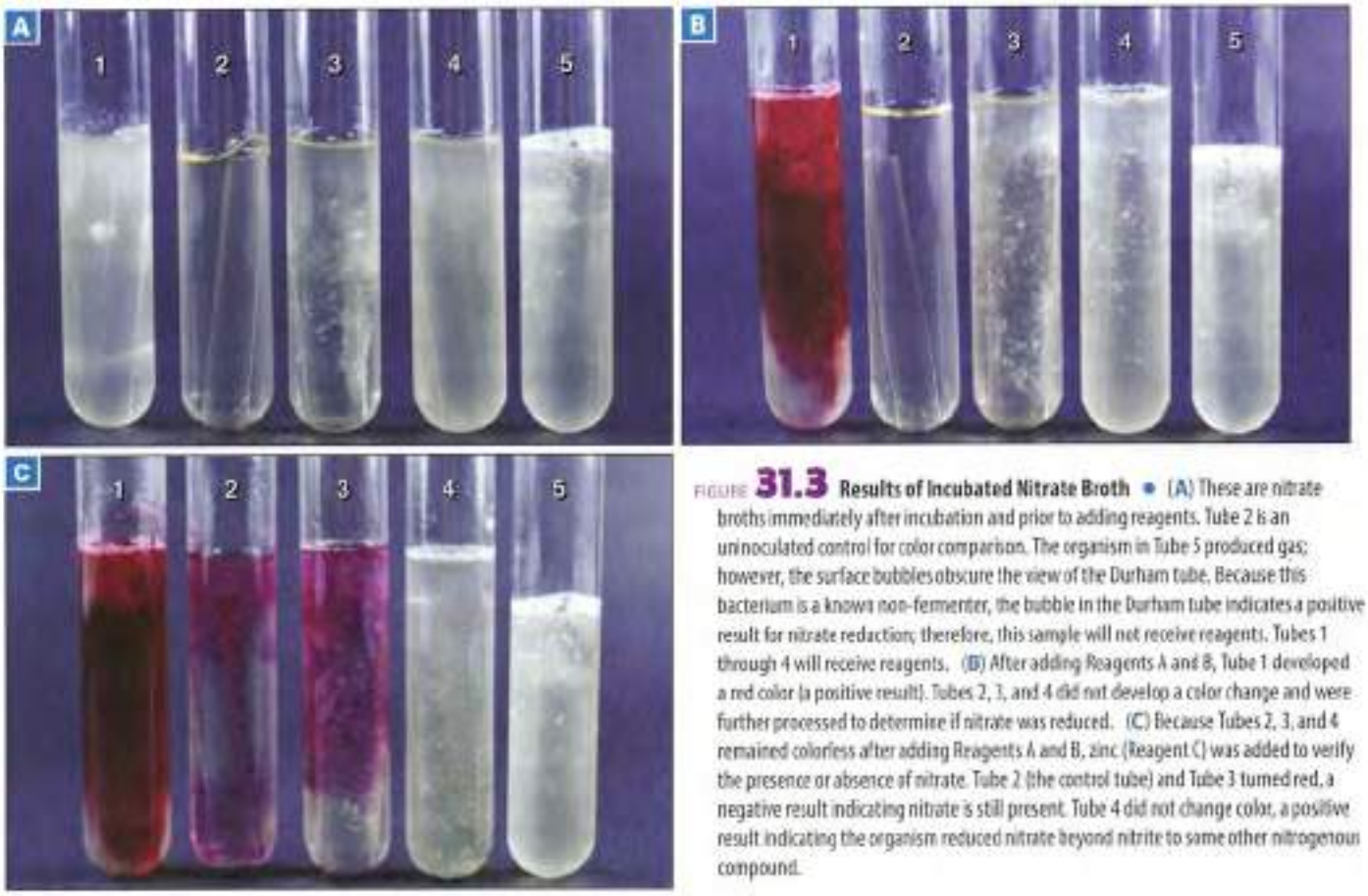


FIGURE **31.3** **Results of Incubated Nitrate Broth** • (A) These are nitrate broths immediately after incubation and prior to adding reagents. Tube 2 is an uninoculated control for color comparison. The organism in Tube 5 produced gas; however, the surface bubbles obscure the view of the Durham tube. Because this bacterium is a known non-fermenter, the bubble in the Durham tube indicates a positive result for nitrate reduction; therefore, this sample will not receive reagents. Tubes 1 through 4 will receive reagents. (B) After adding Reagents A and B, Tube 1 developed a red color (a positive result). Tubes 2, 3, and 4 did not develop a color change and were further processed to determine if nitrate was reduced. (C) Because Tubes 2, 3, and 4 remained colorless after adding Reagents A and B, zinc (Reagent C) was added to verify the presence or absence of nitrate. Tube 2 (the control tube) and Tube 3 turned red, a negative result indicating nitrate is still present. Tube 4 did not change color, a positive result indicating the organism reduced nitrate beyond nitrite to some other nitrogenous compound.

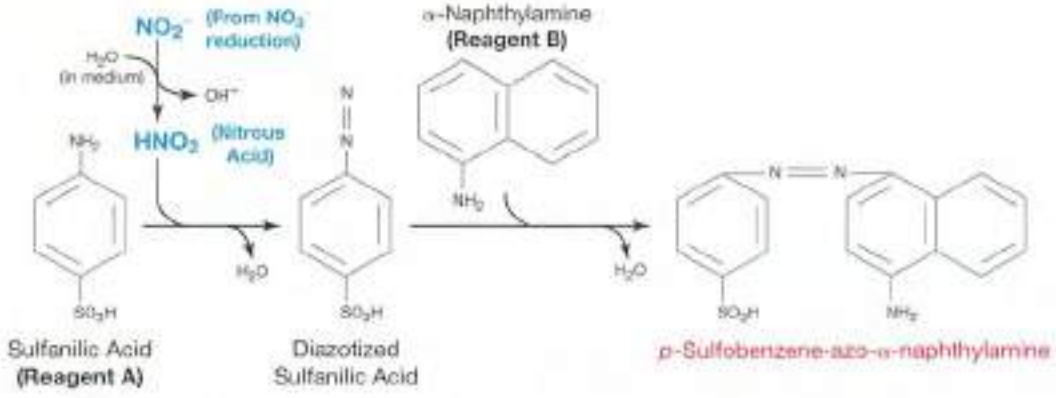


FIGURE **31.4** **Indicator Reaction for Nitrate Reduction to Nitrite** • If nitrate is reduced to nitrite, nitrous acid (HNO_2) will form in the medium. Nitrous acid then reacts with sulfanilic acid (Reagent A) to form diazotized sulfanilic acid, which reacts with the α-naphthylamine (Reagent B) to form *p*-sulfobenzene-azo-α-naphthylamine, which is red. Thus, a red color indicates the presence of nitrite and is considered a positive result for nitrate reduction to nitrite.

Clinical Applications

Virtually all members of *Enterobacteriaceae* perform a one-step reduction of nitrate to nitrite. The nitrate test differentiates them from Gram-negative rods that either do not reduce nitrate or reduce it beyond nitrite to N_2 or other compounds.

The nitrate reduction test is especially useful in clinical laboratories for differentiating *Neisseria gonorrhoeae* (negative for nitrate reduction) from *Kingella denitrificans* (positive for nitrate reduction), a Gram-negative bacterium routinely isolated on selective media for *N. gonorrhoeae*. This test is also useful for differentiating the causative agent of tuberculosis, *Mycobacterium tuberculosis* (positive for nitrate reduction), from similar *Mycobacterium* species that do not perform nitrate reduction.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Nitrate broths (4)
- ❑ Sterile wooden applicators or disposable loops
- ❑ Nitrate test Reagents A and B
- ❑ Zinc powder (Reagent C)
- ❑ Fresh agar slant cultures of these recommended organisms:
  - ☐ *Alcaligenes faecalis*
  - ☐ *Escherichia coli*
  - ☐ *Pseudomonas aeruginosa* (BSL-2) (optional)

Medium and Reagent Recipes

Nitrate Broth

Beef extract	3.0 g
Peptone	5.0 g
Potassium nitrate (KNO_3)	1.0 g
Distilled or deionized water	1.0 L

Adjust to pH 6.8–7.2 at 25°C

Reagent A

Sulfanilic acid	0.8 g
5N acetic acid (30%)	100.0 mL

Reagent B

N,N-Dimethyl-α-naphthylamine	0.6 g
5N acetic acid (30%)	100.0 mL

PROCEDURE

After incubating your inoculated nitrate broths, you should first inspect the Durham tubes in the media for bubbles, an indication of gas production. One or more of the organisms used in the exercise ferment sugars and therefore may produce a gas other than the molecular nitrogen (N_2) made in certain nitrate reduction pathways. To distinguish if the gas made is due to nitrate reduction versus fermentation, you *must* add reagents to all the sample tubes, those where you see a bubble and those where you do not see a bubble. Record any gas observed in the Durham tubes and see if there is a correlation between that and the color results after the reagents and zinc dust have been added. Follow the procedure carefully, and use Table 31.1 as a guide when interpreting the results. Wear a lab coat, gloves, and chemical eye protection when performing this procedure. *Caution!* Use BSL-2 precautions with *Pseudomonas aeruginosa*, if used.

1. **Obtain Your Samples:** Obtain fresh test culture(s) from your instructor. Obtain three—or if *P. aeruginosa* is used, obtain four—nitrate broths. Label two (or three) of them with the names of the organisms, your group name, and the date. Label the third (or fourth) tube "control."
2. **Inoculate the Media:** Use an inoculating loop to inoculate two (or three) broths with the test organisms. Do not inoculate the control.
3. **Incubate Your Samples:** Incubate all tubes at 35±2°C for 24 to 48 hours.
4. **Clean up:** Dispose of the materials as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

→ Next Lab Session

1. **Obtain Your Samples:** Put on your lab safety gear, and then collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Examine Samples for Bubbles in the Durham Tube:** Following the procedural diagram in Figure 31.2, first examine each tube for evidence of gas production. Record your results in the table provided at the end of the exercise. Refer to Table 31.1 when making your interpretations. (Be methodical when recording your results. Red color can have opposite interpretations depending on where you are in the procedure.)

3 **Add Reagents A and B to Each Sample and Make Observations:** Continue using Figure 31.2 as a guide and proceed to the addition of Reagents A and B to *all* tubes. Add 8 drops (approximately 0.5 mL) of Reagent A and then 8 drops (approximately 0.5 mL) of Reagent B to each tube. Mix well, but carefully, and let the tubes stand undisturbed for 10 minutes. Using the control as a comparison, record your test results in the table provided in the data sheet. Set aside any test broths that are positive.

4 **Add Reagent C to Samples That Have Not Developed a Red Color:** Where appropriate, add a very small pinch of zinc dust (Reagent C). You can dip a wooden applicator into the zinc dust and transfer only the amount that clings to the wood. Let the tubes stand for 10 minutes. Record your results in the table provided in the data sheet at the end of this exercise.

5 **Clean up:** Dispose of the materials as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

TABLE **31.1** Nitrate Reduction Test Results and Interpretations •

Result	Interpretation	Symbol
Gas in Durham tube (***Note:*** You can only rely on this measure if you are absolutely certain the tested bacterium does *not* conduct fermentation. If you do not know if the test bacterium performs fermentation, then you must add reagents to assess if nitrate was reduced.)	If you are certain the test bacterium does *not* perform fermentation, then you may conclude denitrification occurred ($NO_3^- \rightarrow NO_2^- \rightarrow N_2$).	+
Red color (after addition of Reagents A and B)	Nitrate reduction to nitrite ($NO_3^- \rightarrow NO_2^-$); nitrate reductase is present	+
No color change (after the addition of Reagents A and B)	Incomplete test; requires the addition of zinc dust	
No color change (after addition of Reagent C, zinc dust)	Nitrate reduction to gaseous (NO, $\frac{1}{2}N_2O$, or $\frac{1}{2}N_2$) or reduction to nongaseous nitrogen compounds such as ammonium (NH_4^+)	+
Red color (after addition of Reagent C, zinc dust)	No nitrate reduction	−

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 31
DATA SHEET

Nitrate Reduction Test

During the Lab

1 Using Table 31.1 as a guide, enter your results and interpretations in the following table. If you have a positive for gas production, look up the fermentation characteristics of that organism in a standard reference, such as ***Bergey's Manual of Determinative Bacteriology*** and consider that information as you shape your interpretation.

Organism	Results			Interpretation (be as specific as possible with respect to end products if positive)
	Gas	Color		
	Y/N	After Reagents	After Zinc	
Uninoculated control				

Lab Related Questions

1 Choose the true statement(s) about nitrate (select all that apply).

a. It can be oxidized to nitrite.

b. It can be reduced to molecular nitrogen (N_2).

c. It can serve as a final electron acceptor in anaerobic respiration.

d. It is present in the media used for this exercise.

e. It can be reduced to ammonium.

2 The nitrate reduction test is performed using:

a. Differential media.

b. Selective media.

c. Enriched media.

d. Defined media.

e. Synthetic media.

3 Reagents A and B are added to:

a. Detect nitrate production.

b. Detect nitrite production.

c. Convert nitrite to nitrate.

d. Convert nitrate to nitrite.

e. Activate nitrate reductase.

4 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ Nitrate reductases convert nitrite to nitrate.

_____ Bacteria that are anaerobic may use nitrate as a final electron acceptor.

_____ Reagent C facilitates the detection of nitrate in the media.

_____ An organism that reduces nitrate to ammonium will turn red after adding Reagents A, B, and C.

5 Number the steps below so they are in the proper order for the nitrate reduction test.

_____ Development of a red color indicates a negative result.

_____ Add Reagent B.

_____ Look for a bubble in the Durham tube.

_____ Inoculate the media.

_____ Development of a red color indicates a positive result.

_____ Add Reagent C if no color developed.

_____ Incubate the media.

_____ Add Reagent A.

6 Assume you see gas in the Durham tube and after adding Reagents A and B, a red color develops. Based on these observations, what can you conclude? (Select all that apply.)

a. The bacterium is an obligate aerobe.

b. The bacterium makes nitrate reductase.

c. The gas observed may be due to a fermentation reaction.

d. The organism is *not Neisseria gonorrhoeae*.

e. You must add zinc to the media before making any conclusions.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 31
DATA SHEET
(continued)

Critical Thinking

1 Suppose you remove your test cultures from the incubator and notice that one of them—a known fermenter—has a gas bubble in the Durham tube. Knowing that fermenters frequently produce gas, you ignore the bubble and proceed to the next step. Adding reagents produces no change, and neither does adding zinc. Based on this information what can you conclude? Be sure to explain your reasoning.

2 Although strict anaerobes may reduce nitrate, the nitrate reduction test as we performed it in this exercise would not determine the nitrate reduction potential of a strict anaerobe. Explain why.

— Lab Notes —

EXERCISE 32

Citrate Utilization Test

After doing this exercise, you should be able to:

1. Explain what the citrate utilization test reveals about a bacterial species.
2. Describe how to perform and interpret the citrate utilization test.
3. State the applications of the citrate utilization test.
4. Perform a citrate utilization test and interpret the test results.

Background

In the following exercise you will perform an example of a utilization test using Simmons citrate medium. **Citrate** is a six-carbon weak organic acid. It's found throughout nature, such as in citrus fruits. It is also an intermediate in the Krebs cycle, and thereby associated with aerobic respiration. Despite citrate being an intermediate in aerobic respiration, the citrate utilization test does not reveal if an organism performs aerobic respiration. Rather, it reveals if an organism can ferment citrate and therefore rely on citrate as its sole carbon source.

While numerous bacterial species make citrate, not all that make it can use it as a sole carbon source. Organisms that can use citrate as their sole carbon source must import the molecule into the cell and then metabolize it. To achieve this, bacteria require **citrate permease** to import citrate from the extracellular environment into the cell. Then, **citrate lyase** (Fig. 32.1) converts the imported citrate into oxaloacetate and acetate. The oxaloacetate is then decarboxylated (CO_2 is removed) by **oxaloacetate decarboxylase** to form pyruvate. The resulting pyruvate may then be fermented to a variety of intermediates. The cell's environmental pH and the availability of certain enzymes influences which fermentation pathway(s) further metabolizes pyruvate. From a metabolic standpoint, it is worth pointing out that the bacteria that thrive on Simmons citrate agar tend to perform fermentation pathways that generate ATP and reducing power (i.e., NADPH or NADH).

Citrate Media Formulation

Simmons citrate agar is a defined solidified agar medium (usually poured in a slant format) where sodium citrate is the only carbon source in the medium and ammonium phosphate serves as an inorganic nitrogen source.

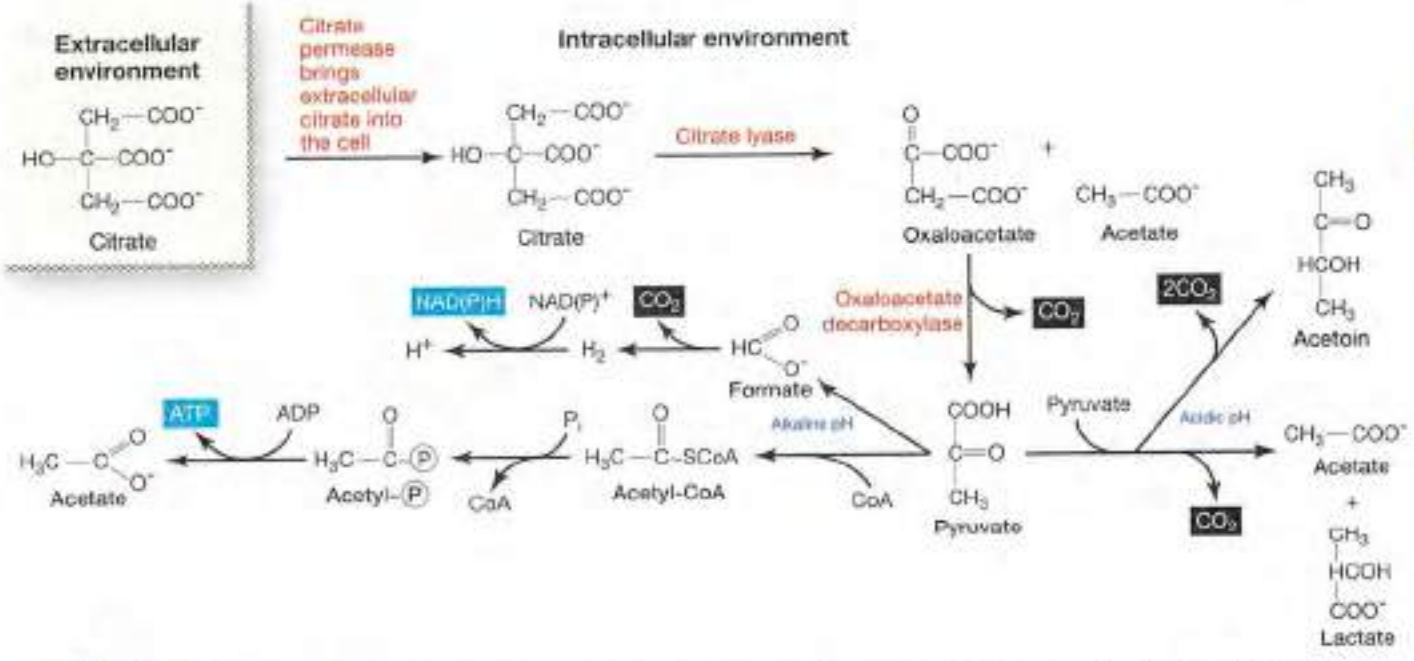


FIGURE **32.1** **Citrate Chemistry** • Citrate permease imports citrate into the cell. From there, citrate lyase converts citrate into oxaloacetate and acetate. Next, the enzyme oxaloacetate decarboxylase performs a decarboxylation reaction in which CO_2 is removed from oxaloacetate thereby forming pyruvate. The resulting pyruvate can be converted to a variety of products depending on the pH of the cell's environment and the enzymes the cell possesses. One option, the formate/acetyl-CoA pathway shown in detail to the left, produces the useful compounds ATP and NADH/NADPH.

Consequently, only bacteria that can survive with citrate as their sole carbon source and use ammonium phosphate as their nitrogen source will grow on this media.

As bacteria grow on the media and metabolize citrate, they also convert ammonium phosphate in the media to ammonia (NH_3) and ammonium hydroxide (NH_4OH). Because NH_3 and NH_4OH are bases, they will alkalinize the agar (raise the media's pH). The media includes a pH indicator dye, bromothymol blue, which allows us to determine if a pH shift to an alkaline state has occurred. The dye appears green at pH 6.9 (the media's original preparation pH) and blue at a pH level of pH 7.6 (the media's alkalinized pH if NH_3 and NH_4OH were made). Thus, conversion of the medium from its original green color to blue means the bacteria are actively growing and indicates a positive citrate test result (Fig. 32.2 and Table 32.1).

Occasionally bacteria will grow on a Simmons citrate slant *without* producing a color change in the media. In most cases, this is because of too short an incubation. In the absence of a color change, growth on the slant suffices as evidence of citrate utilization and thereby constitutes a positive test result. To avoid confusion between actual growth and a heavy inoculum, which may be misinterpreted as growth, citrate slants should be lightly inoculated. One way to decrease the amount of inoculum added to the agar is to use an inoculating needle rather than a loop to streak the agar slant surface. However, if this strategy is employed, you should be careful that you do not gouge the agar surface with the needle.

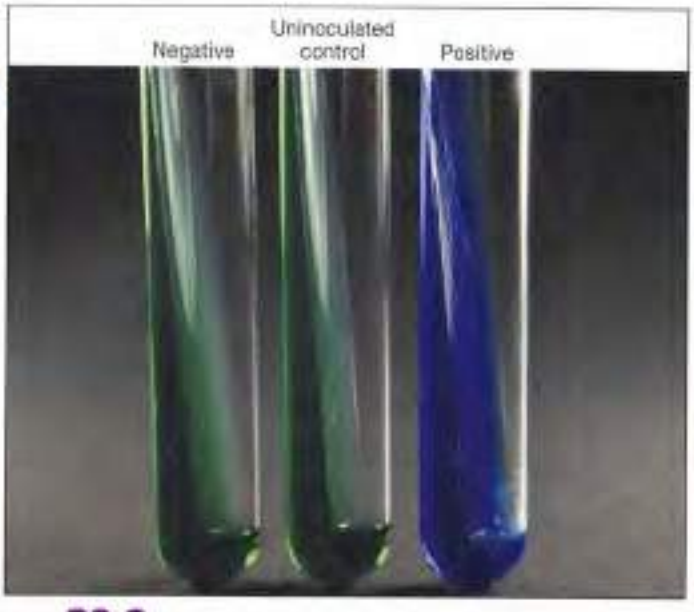


FIGURE **32.2** **Citrate Utilization Test Results** • After incubation, if the media appears green and there is no growth on the slant's surface, then the test result is negative; the bacterium cannot use citrate as its sole carbon source. In contrast, a blue media coloration and the presence of growth indicates a positive test result; the bacterium can use citrate as its sole carbon source.

Interpreting Citrate Utilization Test Results

TABLE **32.1** **Citrate Utilization Test Results and Interpretations** •

Result	Interpretation	Symbol
Media appears blue (even if only a portion of the media turned blue) *and* growth is evident	Citrate is utilized (organism has the enzymes needed to use citrate as its sole carbon source)	+
No color change, but growth is evident	Citrate is utilized (organism has the enzymes needed to use citrate as its sole carbon source)	+
No color change *and no growth*	Citrate is not utilized (organism lacks one or more of the enzymes needed to use citrate as its sole carbon source)	–

Clinical Applications

As previously discussed, identifying metabolic properties is an important means of determining a bacterial isolate's identity. The citrate utilization test reveals if a bacterium can use citrate as its sole carbon source. Citrate utilization is one part of a test series referred to as the IMViC (*I*ndole, *M*ethyl Red, *V*oges-Proskauer and *C*itrate tests) that helps differentiate members of the family *Enterobacteriaceae* from one another. In particular, the citrate test is useful for differentiating certain fecal coliforms from one another. For example, *E. coli* and *Shigella flexneri* do not typically grow on Simmons citrate agar while *Enterobacter aerogenes* does. Recall that a number of fecal coliforms cause gastrointestinal illness and are primarily transmitted via fecal-contaminated food and/or water.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Simmons citrate slants (3)
- ❑ Fresh agar slant cultures of these recommended organisms:
  - ☐ *Enterobacter aerogenes*
  - ☐ *Escherichia coli*

Medium Recipe

Simmons Citrate Agar

Ammonium dihydrogen phosphate ($NH_4H_2PO_4$)	1.0 g
Dipotassium phosphate (K_2HPO_4)	1.0 g
Sodium chloride (NaCl)	5.0 g
Sodium citrate ($C_6H_5O_7Na_3$)	2.0 g
Magnesium sulfate ($MgSO_4$)	0.2 g
Agar	15.0 g
Bromothymol blue	0.08 g
Distilled or deionized water	1.0 L

Adjust to pH 6.7–7.1 at 25°C

PROCEDURE

You will be inoculating Simmons citrate slants with a citrate-positive and a citrate-negative organism. To avoid confusing growth on the slant with a heavy inoculum, be sure to lightly inoculate (your instructor may suggest using a needle instead of a loop to help you reduce how much bacteria you add). Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

1. **Obtain Your Samples:** Obtain three Simmons citrate tubes. Label two with the names of the organisms, your group name, and the date. Label the third tube "control."
2. **Inoculate the Media:** Using an inoculating needle and *light inoculum*, streak the slants with the test organisms. Do not inoculate the control.
3. **Incubate Your Samples:** Incubate all tubes at 35±2°C for 48 hours.
4. **Clean up:** Dispose of stock samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

→ Next Lab Session

1. **Obtain Your Samples:** Put on your lab safety gear, and then collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Examine Your Samples:** Observe each sample for color changes and/or growth on the media.
3. **Record Your Result:** Record your results in the table provided in the data sheet at the end of this exercise.
4. **Clean up:** Dispose of the samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 32

DATA SHEET

Citrate Utilization Test

During the Lab

1 Using Table 32.1 as a guide, enter your results in the following table.

Organism	Color Result	(+ or –)	Interpretation
Control			

Lab Related Questions

1 Choose the true statement(s) about citrate (select all that apply).

a. It is an intermediate of the Krebs cycle.

b. It is a three-carbon compound.

c. It can be converted to oxaloacetate.

d. It raises the media's pH if present.

e. It can be used as a nitrogen source.

2 Which of the following is indicative of a positive test result for citrate utilization? (Select all that apply.)

a. The media appears green and growth is absent.

b. The media appears blue and growth is present.

c. The media appears green and growth is present.

d. The media appears blue and growth is absent.

3 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ Only bacteria that can use citrate as their sole carbon source will grow on Simmons citrate agar.

_____ *E. coli* will typically generate a positive test result when grown on Simmons citrate agar.

_____ This test can determine if a bacterium performs aerobic respiration.

_____ An organism that requires an organic nitrogen source will not grow on Simmons citrate agar.

4 The citrate test determines if a bacterium:

a. Metabolizes citrate.
b. Requires a carbon source.
c. Requires a nitrogen source.
d. Produces acidic metabolic byproducts.
e. Is a pathogen.

5 Which of the following are unlikely to grow on Simmons citrate agar? (Select all that apply.)

a. *E. coli*
b. *S. flexneri*
c. A bacterium that requires an inorganic nitrogen source
d. A bacterium that lacks citrate permease
e. *E. aerogenes*

6 Why may a heavy inoculum be problematic in this test?

a. The medium's pH can be falsely elevated if too many bacteria are growing on the agar.
b. You will not be able to achieve colony isolation.
c. You may introduce a contaminant into the agar.
d. You may mistake the inoculum for actual growth.
e. You may be unable to assess the media's color if the slant is overgrown.

7 Choose the statement(s) that accurately describe(s) Simmons citrate agar (select all that apply).

a. It is considered enriched media.
b. It is considered a defined media.
c. There is an organic source of carbon in the media.
d. Simmons citrate agar contains methylene blue as a pH indicator.
e. After incubation, you must add reagents to this media before interpreting test results.

Critical Thinking

1 It is a Friday afternoon and you head to the lab to inspect the Simmons citrate agar bacterial samples that you inoculated on Wednesday of the same week. The samples were all grown at 37°C. You do not observe any growth in the tube for your test sample nor for the negative control. However, the positive control grew just fine. You decide your tested bacteria cannot utilize citrate as a sole carbon source. Planning to dispose of the samples on Monday, you simply place the samples in a test-tube rack at room temperature in the biosafety hood where they will be safe until your return. When you return to the lab on Monday, you notice that your unknown sample has abundant growth and the media is completely blue. Your negative control sample still does not exhibit growth and remained green. Based on this, what can you conclude about your test specimen? Be sure to explain your reasoning and provide details from the scenario that support your conclusion.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 32
DATA SHEET
(continued)

2 You perform the citrate utilization test and after 48 hours of incubation you make the following observations. Should you trust your findings? Be sure to explain your reasoning.

Sample Description	Media Color after 48 Hours of Incubation	Growth after 48 Hours of Incubation
E. coli	Blue	Present
E. aerogenes	Blue	Present
Unknown sample 2	Blue	Present

— Lab Notes —

EXERCISE 33

Amino Acid Decarboxylation (Decarboxylase Tests)

After doing this exercise, you should be able to:

1. Explain what amino acid decarboxylation tests reveal about a bacterial species.
2. Describe how to perform and interpret amino acid decarboxylation tests.
3. State the applications of amino acid decarboxylation tests.
4. Perform amino acid decarboxylation tests and interpret the test results.

Background

Amino acids are the building blocks of protein, but when resources are limited, some bacteria use them as a carbon and energy source. Using amino acids for purposes other than making proteins generally involves removing one or both of their functional groups: the amine (NH_2) by a **deaminase** and/or the carboxylic acid (COOH) group using a **decarboxylase enzyme.**

In this exercise, you will examine the ability of bacteria to decarboxylate the amino acid lysine using the enzyme lysine decarboxylase. As optional procedures, you may also test for the presence of ornithine decarboxylase and arginine decarboxylase in the assigned bacteria. (*Note:* Each decarboxylase is specific to its own amino acid substrate.) The indole test, discussed in Exercise 36, relies on a bacterium's ability to deaminate an amino acid.

Møller's Decarboxylase Medium Formulation

To prepare the media for the decarboxylase tests, a single amino acid is added to a foundational medium called Møller's decarboxylase base medium. The base medium contains beef extract and peptone as nitrogenous nutrients for bacteria; glucose serves as the fermentable carbohydrate; and pyridoxal is a necessary cofactor for decarboxylase enzymes to function. The type of amino acid added to the base medium depends on which decarboxylase you are aiming to detect. For example, if you want to determine if your test bacterium has the enzyme lysine decarboxylase, then the base media would be supplemented with the amino acid lysine. The dye bromocresol purple serves as the pH indicator in the media. It appears yellow below pH 5.2 and purple at pH 6.8 or higher.

Performing and Interpreting Amino Acid Decarboxylation Tests

After inoculation, an overlay of mineral oil is added to seal the medium from the air. This ensures the surface of the media's pH is not artificially raised (alkalized) through interactions with the ambient air and it helps to maintain a lower oxygen environment that favors glucose fermentation (Fig. 33.1). Recall that glucose fermentation tends to lead to an accumulation of acidic end products; all *Enterobacteriaceae* family members ferment glucose to acidic end products. A low pH combined with the presence of a specific amino acid induces **decarboxylase-positive** organisms to produce the enzyme that can **catabolize** (break down) the given amino acid. That is, the specific decarboxylase gene is "switched on."

Decarboxylation of the amino acid results in accumulation of amines, which are alkaline (Fig. 33.2). As amines accumulate, the resulting alkaline pH causes the pH indicator, bromocresol purple, to appear purple; this is a positive test result (Fig. 33.3, left). If the organism is a glucose fermenter but does not produce the appropriate

FIGURE **33.1** **Adding Sterile Mineral Oil to the Tubes** • Gently let the oil slide down the side of the test tube to a depth of approximately 4 mm (not mL). It is important to keep the mineral oil sterile to prevent contamination.

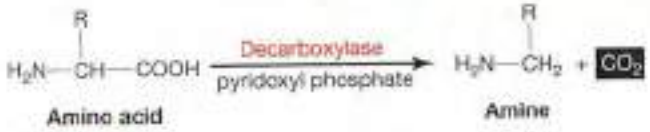


FIGURE **33.2** **Amino Acid Decarboxylation** • Removal of an amino acid's carboxyl group results in the formation of an amine and carbon dioxide.

decarboxylase, the medium will appear yellow (Fig. 33.3, middle). If the organism does not ferment glucose, the medium does not exhibit a color change (Fig. 33.3, right).

Different amino acids can be added to the base broth. The three amino acids most frequently used in decarboxylase test media are lysine, ornithine, and arginine. Figures 33.4 through 33.6 show the chemistry associated with decarboxylating each of these amino acids. Although the chemistry slightly differs based on the amino acid catabolized, positive and negative test results are interpreted in the same manner as used for the lysine decarboxylase test (Table 33.1).

Lastly, it is worth noting that arginine decarboxylase medium can not only reveal if a bacterium makes arginine decarboxylase, but it can also detect the presence of a second pathway for arginine catabolism, the arginine dihydrolase system. In this system, arginine is converted to citrulline and then into ornithine, ATP, CO_2, and two NH_3 (ammonia) (Fig. 33.7). The generated ammonia raises the pH and turns the medium purple, just as would occur if arginine decarboxylase were present.

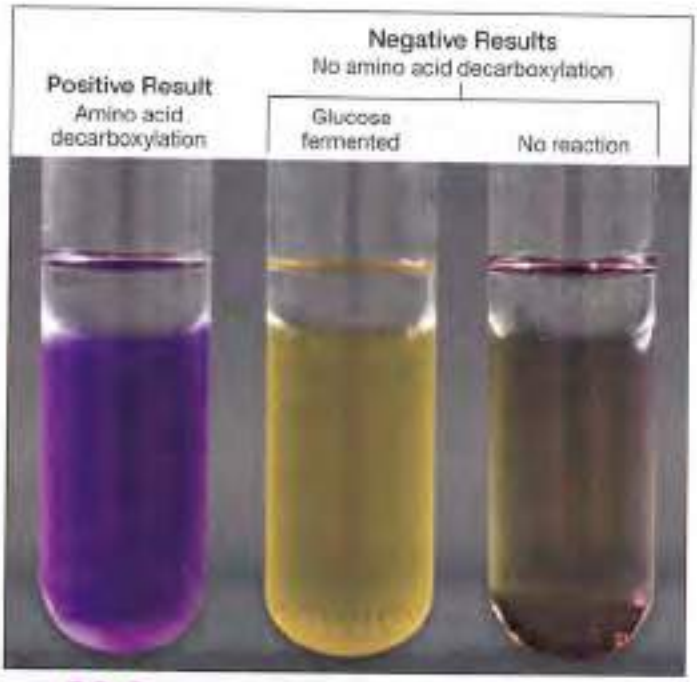


FIGURE 33.3 Decarboxylation Test Results • (**Left**) A lysine decarboxylase-positive result appears purple; (**Middle**) a lysine decarboxylase-negative result with glucose fermentation appears yellow; (**Right**) a decarboxylase-negative result without fermentation appears a murky yellow/tan color, the same color as your uninoculated control, which is not shown here. The color results shown here are representative of other decarboxylase media as well.

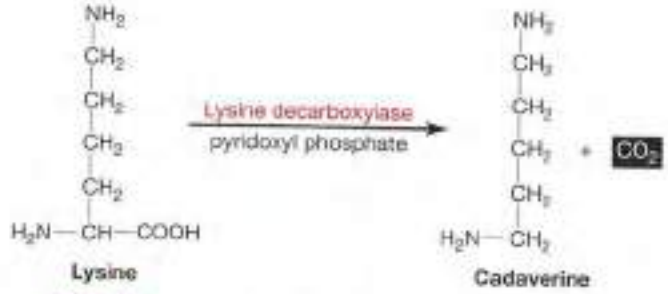


FIGURE 33.4 Lysine Decarboxylation • Decarboxylation of the amino acid lysine produces cadaverine and CO_2.

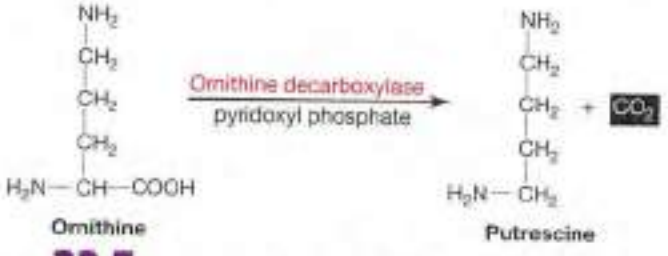


FIGURE 33.5 Ornithine Decarboxylation • Decarboxylation of the amino acid ornithine produces putrescine and CO_2.

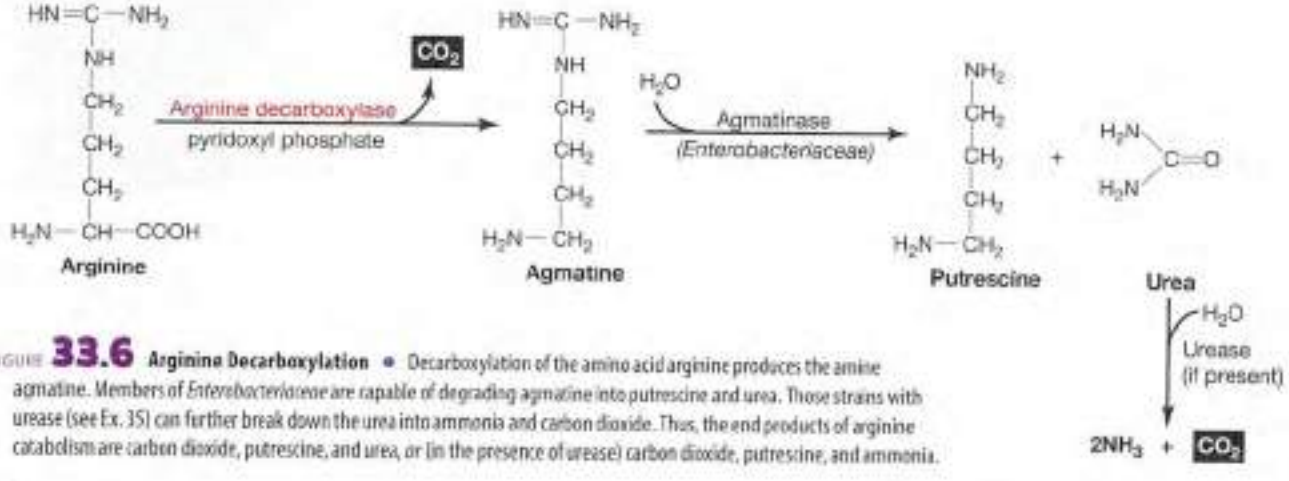


FIGURE 33.6 Arginine Decarboxylation • Decarboxylation of the amino acid arginine produces the amine agmatine. Members of *Enterobacteriaceae* are capable of degrading agmatine into putrescine and urea. Those strains with urease (see Ex. 35) can further break down the urea into ammonia and carbon dioxide. Thus, the end products of arginine catabolism are carbon dioxide, putrescine, and urea, or (in the presence of urease) carbon dioxide, putrescine, and ammonia.

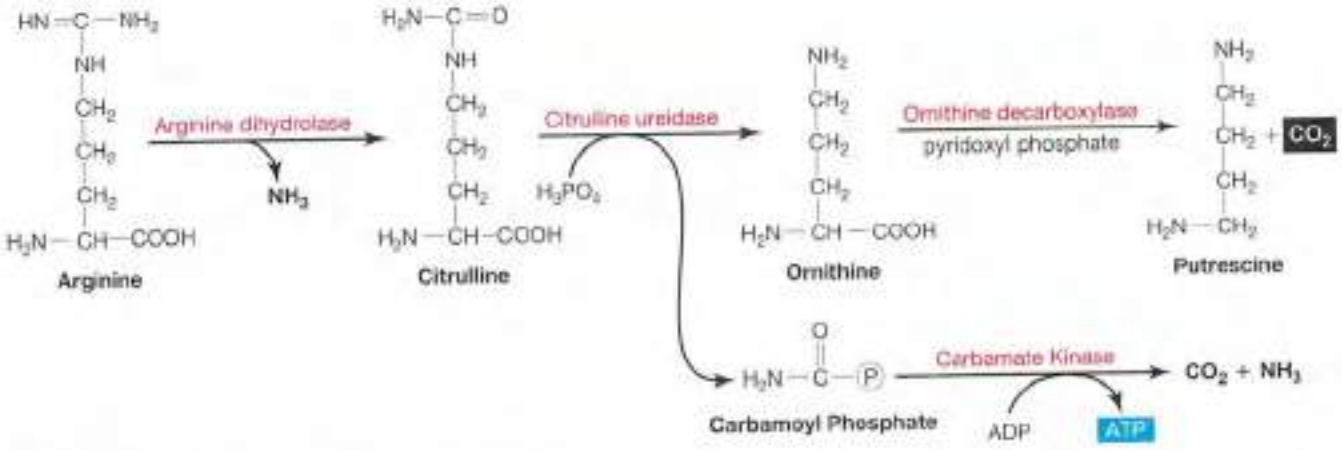


FIGURE **33.7** **Arginine Dihydrolase System** • In addition to decarboxylation, arginine may also be catabolized by a dihydrolase enzyme with the production of ornithine, NH_3, and CO_2. In ornithine decarboxylase-positive organisms, the ornithine is further degraded into putrescine and CO_2. The example shown here is a dihydrolase system of *Pseudomonas putida* in which ATP synthesis occurs. Regardless of the specifics, the arginine dihydrolase pathway is indistinguishable from the arginine decarboxylase pathway because both produce alkaline end products.

TABLE **33.1** **Decarboxylase Test Results and Interpretations** •

Result	Interpretation	Symbol
No color change	No decarboxylation of the amino acid (insert amino acid name)*: the specific decarboxylase is absent or products are not detectable	−
Yellow	Fermentation of glucose; no decarboxylation of the amino acid (insert amino acid name)*: the specific decarboxylase is absent or products are not detectable	−
Purple (may be slight)	Decarboxylation of the amino acid (insert amino acid name)*: organism produces the specific decarboxylase enzyme	+

* The amino acid included in the media can vary. Therefore, all interpretations must be described as they relate to the specific amino acid in the media. Example: If using lysine decarboxylase media, the amino acid in the media is lysine.

6

Clinical Applications

The decarboxylase tests are a form of utilization test that helps microbiologists identify bacterial species. Decarboxylase media can include any one of several amino acids, with the most frequently used amino acids being lysine, arginine, and ornithine. Typically, decarboxylase media are used to differentiate organisms in the family *Enterobacteriaceae* and to distinguish them from other Gram-negative rods.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Lysine decarboxylase broths[1] (3)
- ❑ Tubes (3) of the Møller's decarboxylase base media (media lacking the test amino acid)
- ❑ Sterile mineral oil in microtubes
- ❑ Sterile transfer pipettes (16)
- ❑ Fresh agar slant cultures of these recommended organisms:
  - ☐ *Enterobacter aerogenes*
  - ☐ *Providencia stuartii*
  - ☐ *Enterobacter cloacae* (optional, see footnote)

[1] This exercise assumes that only two bacterial species will be tested and that only lysine decarboxylase media will be used. However, if desired, arginine and ornithine decarboxylase media may also be used in this exercise. If these other decarboxylase test media are included, it is recommended that you also include *Enterobacter cloacae* as one of the test organisms.

Medium Recipe

Lysine Decarboxylase Medium (Møller)[2]

Peptone	5.0 g
Beef extract	5.0 g
Glucose (dextrose) ($C_6H_{12}O_6$)	0.5 g
Bromocresol purple	0.01 g
Cresol red	0.005 g
Pyridoxal	0.005 g
L-Lysine ($C_4H_7COOH(NH_2)_2$)	10.0 g
Distilled or deionized water	1.0 L

Adjust to pH 5.8–6.2 at 25°C

[2] Note: The base medium used for your controls is the same as the lysine decarboxylase medium, except lysine is not added.

PROCEDURE

This exercise assumes you will test two different bacterial species for their ability to decarboxylate the amino acid lysine. Please note, your instructor may modify the number and/or type of bacteria tested. They may also alter which decarboxylases test(s) is/are performed. That said, positive and negative results—with and without acid production—look the same regardless of the amino acid in the decarboxylase medium. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

1. **Obtain Your Samples and Media:** Obtain the two bacterial stock cultures that you are going to test. You will also need three tubes of Møller's decarboxylase base medium (media lacking the test amino acid) and three tubes of lysine decarboxylase media (the media that contains the test amino acid). *Note:* Your instructor may modify the number of tubes and the type of decarboxylase media you use, so pay attention and adapt your process accordingly.
2. **Label the Media Tubes:**
   a. **Label the Three Tubes of Base Media that Lack Lysine:** One tube will serve as your uninoculated control; the other two tubes are to grow the two test bacteria. Label each tube with your name, the date, the sample description (i.e., the bacterium's name or uninoculated), and the media's name.
   b. **Label the Three Tubes of Lysine Decarboxylase Media:** One tube will serve as your uninoculated control; the other two tubes are to grow the two test bacteria. Label each tube with your name, the date, the sample description (i.e., the bacterium's name or uninoculated), and the media's name.
3. **Inoculate the Media:** Use an inoculating loop to inoculate a separate lysine decarboxylase broth for each test bacterium. Repeat the process for the base media. As a reminder, do not inoculate the tubes you labeled "uninoculated."
4. **Mineral Oil Overlay:** Aseptically add mineral oil to each tube (Fig. 33.1). Be careful not to introduce contaminants while adding the oil.
5. **Incubate Your Samples:** Incubate all tubes at 35±2°C for 96 hours (4 days) up to a full week.
6. **Clean up:** Dispose of stock samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

→ Next Lab Session

1. **Obtain Your Samples:** Put on your lab safety gear, and then collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Examine Your Samples:** Observe each sample for color changes. Refer to Figure 33.3 and Table 33.1 when interpreting your results. If the inoculated base medium without the added amino acid becomes alkaline during incubation, the test is considered invalid.
3. **Record Your Result:** Record your results in the table provided in the data sheet at the end of this exercise.
4. **Clean up:** Dispose of the samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 33

DATA SHEET

Amino Acid Decarboxylation (Decarboxylase Tests)

During the Lab

1 Using Table 33.1 as a guide, enter your results in the following table.

Organism	Results				Interpretation
	Base		Lysine		
	Color	+/–	Color	+/–	
Control					

Lab Related Questions

1 Choose the true statement(s) (select all that apply).

a. If an organism has lysine decarboxylase, it can make lysine.
b. If an organism has lysine decarboxylase, it can catabolize lysine.
c. If an organism has lysine decarboxylase, it can deaminate lysine.
d. If an organism has lysine decarboxylase, it will produce putrescine.
e. If an organism has lysine decarboxylase, it will produce CO_2.

2 Which of the following is (are) indicative of a negative test result for amino acid decarboxylation? (Select all that apply.)

a. The media appears yellow.
b. The media appears purple.
c. The media did not manifest a color change.
d. The media appears yellow and cloudy.
e. The media appears blue.

3 Why is mineral oil added in the amino acid decarboxylase test? (Select all that apply.)

a. It protects the samples from contamination.
b. It serves as a carbon source for the bacteria.
c. It promotes glucose fermentation.
d. It acidifies the media.
e. It prevents artificial alkalization of the media.

4 You observe your inoculated sample at Day 3 and notice it is yellow. By Day 6, it is purple. What can you conclude about the organism? (Select all that apply.)

a. It ferments glucose.
b. It made lysine.
c. It cannot ferment glucose.
d. It catabolized lysine.
e. It cannot catabolize lysine.

5 A typical decarboxylase enzyme removes _____ from an amino acid to produce _____ and _____.

6 The pH of media in a positive amino acid decarboxylase test result is:

a. Purple.
b. Basic.
c. Neutral.
d. Acidic.
e. Yellow.

7 In this experiment, the Møller's decarboxylase base medium serves as a:

a. Positive control.
b. Negative control.
c. Practice sample.
d. Back-up sample.
e. Calibration sample.

Critical Thinking

1 How would you modify the test media to determine if a bacterium that ferments lactose can decarboxylate lysine?

2 Think about the effect of the mineral oil on the test results. Would omitting the sterile mineral oil affect either the specificity or the sensitivity of the test, or both? Explain.

EXERCISE 34

Starch Hydrolysis (Amylase Test)

After doing this exercise, you should be able to:

1. Explain what the amylase test reveals about a bacterial species.
2. Describe how to perform and interpret the starch hydrolysis test.
3. State the applications of the starch hydrolysis test.
4. Perform a starch hydrolysis test and interpret the test results.

Background

The starch hydrolysis test (or amylase test) reveals if a bacterium can break down the complex polysaccharide starch. **Starch** is essentially a string of α-glucose molecules bonded together. There are two general versions of this polymer, a linear form called **amylose** and a branched form called **amylopectin**. In most cases, starch exists as a mixture of these two forms, with amylopectin predominating.

Starch is too large to pass through the bacterial cell membrane to be metabolized. Therefore, it must first be broken down extracellularly. This is accomplished by three enzymes: **α-amylase**, **β-amylase**, and **α-1,6-glucosidase**. These enzymes are all secreted extracellularly and are therefore considered **exoenzymes**. Furthermore, because all three enzymes use water to split complex molecules, they are called **hydrolytic** enzymes. Although these enzymes have the aforementioned features in common, they have different roles in breaking down starch into smaller molecules.

The enzyme α-amylase breaks amylose into the monosaccharide α-glucose and the disaccharide α-maltose, whereas β-amylase hydrolyzes amylopectin to form β-maltose and **limit dextrin**, a small branched carbohydrate molecule. Because neither α nor β amylases can disassemble the glucose subunits around branch points of limit dextrin, **α-1,6-glucosidase** breaks down limit dextrin into short, linear oligosaccharides that α-amylase then hydrolyzes to α-glucose and α-maltose (Fig. 34.1).[1]

[1] The carbons of organic molecules are numbered. A "1,4 linkage" is a covalent bond between the first carbon of one glucose and the fourth carbon of another glucose. A "1,6 linkage" is a covalent bond between the first and sixth carbons of two glucose molecules. The "α" and "β" refer to whether the two glucose molecules have the same orientation (α) or are "flipped" 180° relative to one another (β).

Starch Medium Formulation and Test Result Interpretation

Starch agar is a plated medium of beef extract, soluble starch, and agar. When organisms that produce α-amylase, β-amylase, and oligo-1,6-glucosidase are grown on starch agar, they hydrolyze the starch in the area surrounding their growth. Because both starch and its sugar subunits are virtually invisible in the medium, the reagent iodine is used to detect the presence or absence of starch in the vicinity of the bacterial growth. Iodine reacts with starch and produces a blue or dark-brown color. If the bacterium hydrolyzed starch, the added iodine will not encounter starch to react with and therefore you will observe a clear zone around the bacterial growth (Fig. 34.2). Table 34.1 summarizes interpretations for possible test results.

TABLE 34.1 Starch Hydrolysis Test Results and Interpretations

Result	Interpretation	Symbol
Clearing around growth	α-amylase, β-amylase, and/or α-1,6-glucosidase is present (or more simply, amylase is present)	+
No clearing around growth	α-amylase, β-amylase, and α-1,6-glucosidase are absent; (or more simply, amylase is absent or not detectable)	–

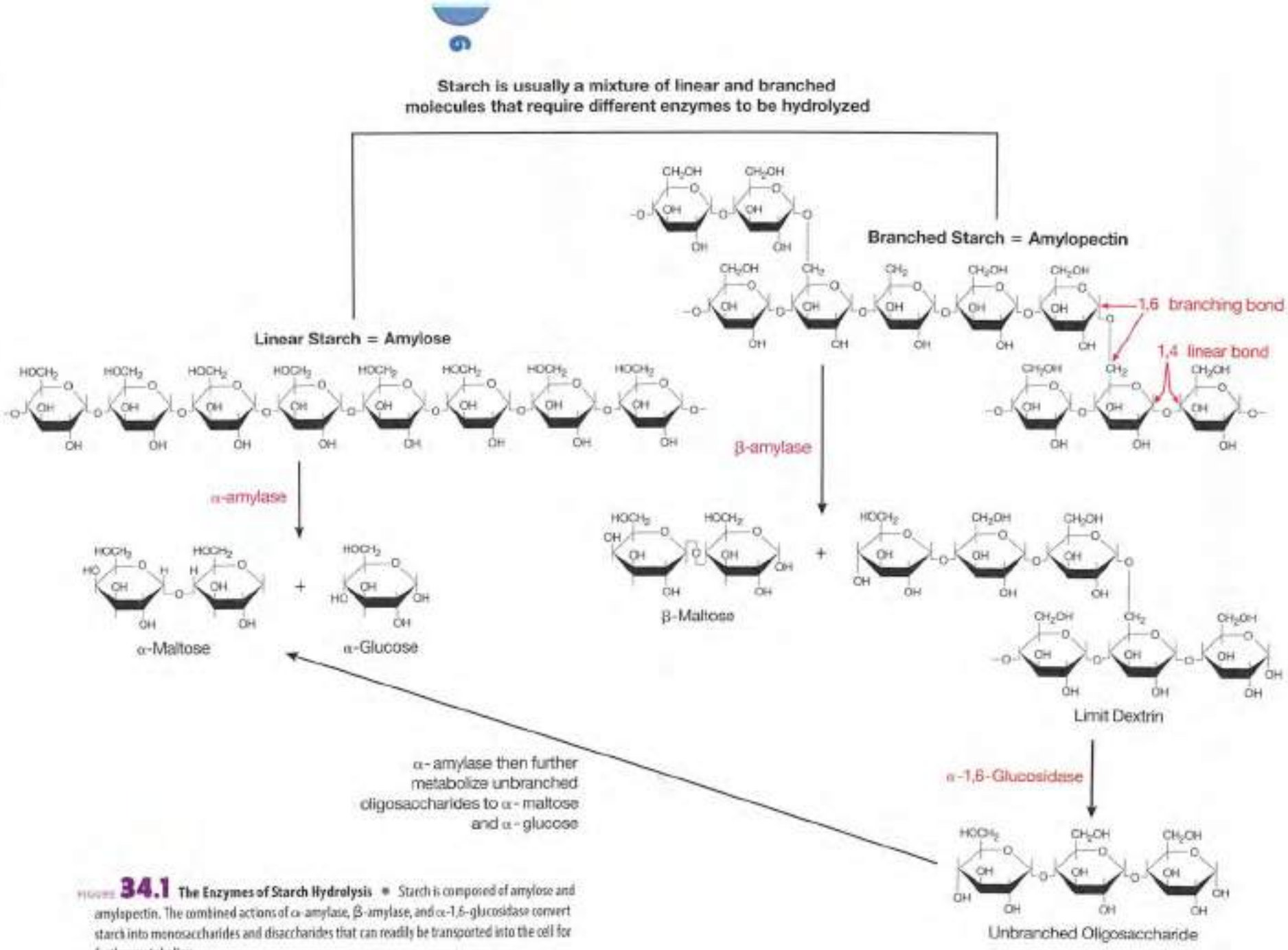


FIGURE 34.1 The Enzymes of Starch Hydrolysis • Starch is composed of amylose and amylopectin. The combined actions of α- amylase, β- amylase, and α-1,6-glucosidase convert starch into monosaccharides and disaccharides that can readily be transported into the cell for further metabolism.

FIGURE **34.2** **Starch Hydrolysis Test Results** • (A) This is an image of a double inoculated starch agar plate before adding iodine. Notice the wavy margins of the bacterial growth. (B) This is the same plate pictured in Panel A, but with iodine added. The clearing in the medium around the organism on the left demonstrates a positive result for starch hydrolysis. The organism on the right, which lacks a clearing, is negative. Notice that the wavy margin of the negative organism's growth appears lighter and could be misinterpreted as a positive result. Drawing a line around the edges of growth prior to adding iodine can help avoid this sort of misinterpretation.

Clinical Applications

Starch agar was originally designed for cultivating *Neisseria*, a bacterial genus that includes *N. gonorrhoeae*, the causative agent of the sexually transmitted infection gonorrhea. However, this medium is no longer used for *Neisseria* isolation. Instead a modified formulation, which has pH indicators added, is useful for isolating and presumptively identifying *Gardnerella vaginalis*, bacteria associated with bacterial vaginosis. It also aids in differentiating members of the genera *Corynebacterium*, *Clostridium*, *Clostridioides*, *Bacillus*, *Bacteroides*, *Fusobacterium*, and *Enterococcus*, most of which include amylase-positive and amylase-negative species.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Starch agar (SA) plate (1)
- ❑ Gram's iodine (from your Gram stain kit)
- ❑ Fresh broth cultures of these recommended organisms:
  - ☐ *Bacillus subtilis*
  - ☐ *Escherichia coli*

Medium Recipe

Starch Agar (SA)

Beef extract	3.0 g
Soluble starch	10.0 g
Agar	12.0 g
Distilled or deionized water	1.0 L

Adjust to pH 7.3–7.7 at 25°C

PROCEDURE

You will inoculate a starch agar plate with two organisms. After incubation, you will add a few drops of iodine to determine whether the tested bacteria hydrolyzed starch. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

1 **Obtain Your Samples and Media:** Obtain the two bacterial stock cultures that you are going to test. You will also need one plate of starch agar.

2. **Label the Starch Plate:** Using a marking pen, divide the starch agar plate into three equal sectors. Label one section for each test bacterium and the third section as an uninoculated control section. Be sure to mark on the bottom of the plate, not the lid. Also, write your name and the date on your plate.
3. **Inoculate the Media:** Use an inoculating loop to inoculate each test bacterium in a zigzag pattern in its designated section. Be careful to keep your inoculation well within each section's boundaries. Leave the control section uninoculated.
4. **Incubate Your Samples:** Incubate all tubes at 35±2°C for 24 hours. If incubated longer, you run the risk of one organism overgrowing the plate and obscuring the results of the other. If the next lab session is more than 24 hours after inoculation, then at the 24-hour mark, the plates can be transferred from the incubator to a refrigerator and maintained at 4°C until your next lab meeting.
5. **Clean up:** Dispose of stock samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

→ Next Lab Session

1. **Obtain Your Samples:** Put on your lab safety gear, and then collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Indicate the Growths' Margins:** Remove the plate from the incubator, and then note the location and appearance of the growth. Use a marking pen to indicate the margins of the growth.
3. **Add Iodine:** Cover the growth and surrounding areas with Gram's iodine. Do not flood the plate, but do add enough iodine so that the agar is completely colorized. *Allow the iodine to soak in.*
4. **Examine Your Samples:** Examine the areas surrounding the growth for clearing. ***Note:*** Usually the growth on the agar prevents contact between the starch and the iodine so no color reaction takes place at that point. Beginning students sometimes look at this lack of color change and judge it incorrectly as a positive result. Therefore, when examining the agar for clearing, look for a halo *around* the growth, not at the growth itself.
5. **Record Your Result:** Refer to Figure 34.2 and Table 34.1 when interpreting your results. Record your results in the table provided in the data sheet at the end of this exercise.
6. **Clean up:** Dispose of the samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 34
DATA SHEET

Starch Hydrolysis (Amylase Test)

During the Lab

1 Using Table 34.1 and Figure 34.2 for guidance, enter your findings in the following table.

Organism	Result	(+/–)	Interpretation (include enzyme)
Control sector			

Lab Related Questions

1 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ The media was originally designed for cultivating *Gardnerella species*.

_____ Test results are visible only after adding iodine.

_____ Iodine reacts with glucose in the media.

_____ The uninoculated region of the plate should appear clear at the end of the experiment.

2 For a bacterium to generate a positive test result in the starch hydrolysis test, which of the following must be present? (Select all that apply.)

a. α-amylase must be present.
b. β-amylase must be present.
c. α-1,4-glucosidase must be present.
d. β-1,6-glucosidase must be present.
e. α-1,6-glucosidase must be present.

3 What serves as the differentiation aspect in this test?

a. The presence or absence of growth on the media.
b. A change in media pH following starch hydrolysis.
c. A media color change during incubation.
d. A color change in the bacterial culture during incubation.
e. The color of the media in the presence of iodine.

4 Choose the true statement(s) about β-amylase (select all that apply).

a. It breaks α 1,6 linkages between glucose molecules.
b. It breaks α 1,4 linkages between glucose molecules.
c. It generates α-glucose.
d. It generates β-maltose.
e. It acts intracellularly.

5 Choose the true statement(s) about α-amylase (select all that apply).

a. It is an exoenzyme.
b. It is a hydrolytic enzyme.
c. It breaks α 1,6 linkages between glucose molecules.
d. It reacts with iodine.
e. It breaks α 1,4 linkages between glucose molecules.

6 How would observing the test results at 12 hours likely affect the experimental outcome? (Select all that apply.)

a. You would be less likely to observe a positive test result.
b. You would be less likely to observe a negative test result.
c. You would be more likely to observe a false-negative test result.
d. You would be more likely to observe a positive test result.
e. You would be more likely to obtain a false-positive result.

7 How would adding glucose to the medium affect the experimental outcome?

a. The bacteria would be more likely to grow, thereby generating a false-positive result.
b. Bacteria would metabolize glucose over starch, generating a false-negative test result.
c. The added glucose would bind to the iodine, generating a false-positive result.
d. The added glucose would inhibit growth, thereby generating a false-negative result.
e. Bacteria would ferment the glucose, thereby generating a false-positive result.

Critical Thinking

1 Suppose you could selectively prevent production of α-amylase or α-1,6-glucosidase in an organism that normally hydrolyzes starch. Which enzyme would the organism miss the most and why?

2 Design an experiment that would allow you to determine if an isolated bacterium has α-amylase and β-amylase, but lacks α-1,6-glucosidase. (***Hint***: You only need the starch hydrolysis test to do this, although it should be modified.)

EXERCISE 35

Urea Hydrolysis (Urease Test)

After doing this exercise, you should be able to:

1. Explain what the urea hydrolysis test reveals about a bacterial species.
2. Describe how to perform and interpret the urea hydrolysis test.
3. State the applications of the urea hydrolysis test.
4. Perform a urea hydrolysis test and interpret the test results.

Background

This test reveals if a bacterium produces the intracellular hydrolytic enzyme **urease**, which uses water to catabolize (break down) urea to form ammonia and carbon dioxide (Fig. 35.1). **Urea** is produced as a byproduct of amino acid catabolism and is the primary nitrogenous waste in the urine of many land animals (including mammals). Although urea is present at high levels in urine, it is not exclusively present in urine; it is also present in blood, sweat, and even in the stomach.

Because urease generates an alkaline byproduct (ammonia), the enzyme can serve as a virulence factor for some pathogens by counteracting acid in the microbe's environment. For example, *Helicobacter pylori* (the bacterium that causes stomach ulcers) uses urease to survive in the acidic stomach environment. *Streptococcus salivarius*, an oral commensal, also produces urease. Furthermore, many enteric bacteria will metabolize urea if it is present and another usable nitrogen source is absent.

Urea Medium Formulation and Test Result Interpretation

The original urease test used urea agar, which was formulated to differentiate enteric organisms capable of rapid urea hydrolysis (rapid urease-positive bacteria, such as *Proteus*, *Morganella morganii*, and some *Providencia stuartii* strains) from slower urease-positive and urease-negative bacteria. A pH indicator was used to track the rising pH due to accumulation of ammonia. Rapid urease-positive organisms produce a positive result within a day; slower urease-positive organisms may take several days to produce a readable result.

In this exercise, you will be using urea broth, which differs from urea agar in two important ways. First, the only nutrient source other than urea is a trace level (0.0001%) of yeast extract. Second, it contains buffers strong enough to inhibit alkalinization of the medium by all but the rapid urease-positive organisms mentioned previously. The pH indicator phenol red, which is yellow or orange below pH 8.4 and red or pink above, is included to expose any increase in pH. The development of a pink color in the medium in 24 hours or less indicates a rapid urease-positive organism. If the media appears orange or yellow, the test result is deemed negative (Fig. 35.2 and Table 35.1).

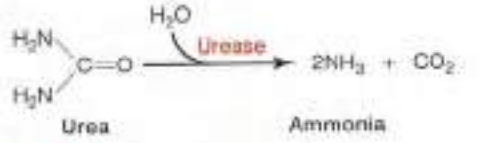


FIGURE 35.1 Urea Hydrolysis • Urea hydrolysis produces ammonia, which raises the pH in the medium and turns the pH indicator pink.

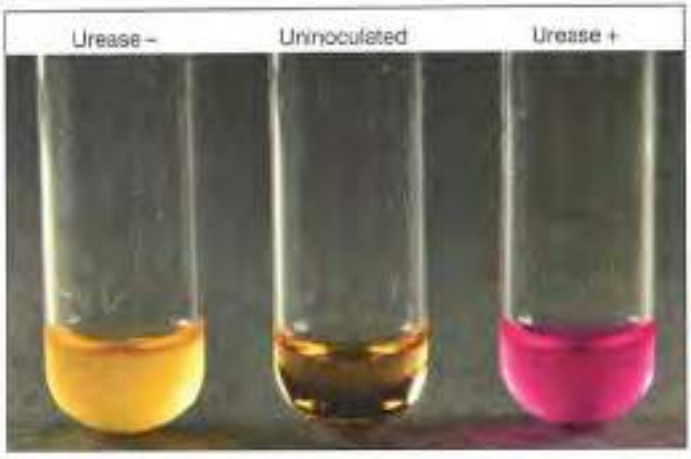


FIGURE 35.2 Urea Hydrolysis Test Results • These are urea broth tubes after a 24-hour incubation. Urease-positive samples will appear bright pink after incubation. Urease-negative samples remain yellow.

TABLE 35.1 Urea Hydrolysis Test Results and Interpretations •

Result	Interpretation	Symbol
Orange or yellow	No urea hydrolysis; urease is absent or not produced in enough quantity to produce results in 24 hours; or the organism cannot live in urease broth	–
Pink	Rapid urea hydrolysis; urease was produced	+

Clinical Applications

Various pathogenic bacteria make urease as a virulence factor to survive in host tissues; the presence of urease is therefore an important marker of a number of bacterial infections. For example, common urinary tract pathogens such as *Staphylococcus saprophyticus* and *Proteus* species are rapid hydrolyzers of urea; thus, the urease test readily differentiates these bacteria from urease-negative pathogens like *E. coli*, which are also a common cause of urinary tract infections. The urease test also helps microbiologists identify *H. pylori*, which is associated with gastric and duodenal ulcers, as well as stomach cancer.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Urea broths (3)
- ❑ Sterile wooden applicators or sterile disposable loops (3)
- ❑ Fresh agar slant cultures of these recommended organisms:
  - ☐ *Escherichia coli*
  - ☐ *Proteus vulgaris* (BSL-2)

Medium Recipe

Rustigian and Stuart's Urea Broth

Yeast extract	0.1 g
Potassium phosphate, monobasic (KH_2PO_4)	9.1 g
Potassium phosphate, dibasic (K_2HPO_4)	9.5 g
Urea ($CO(NH_2)_2$)	20.0 g
Phenol red	0.01 g
Distilled or deionized water	1.0 L

Adjust to pH 6.6–7.0 at 25°C

PROCEDURE

You will perform the urease test with urea broths, which should not be read beyond 24 hours of incubation. If your labs are scheduled more than 24 hours apart, arrange to have someone place your broths in a refrigerator until you can examine them. Wear a lab coat, gloves, and chemical eye protection when performing this procedure. *Caution!* **Follow BSL-2 precautions if using *P. vulgaris*.**

1. **Obtain Your Samples and Media:** Obtain the two bacterial stock cultures that you are going to test and three tubes of urease broth medium. Label two with the names of the organisms, your group name, and the date. Label the third tube "control."
2. **Inoculate the Media:** Heavily inoculate the two urea broths with the test organisms. Do not inoculate the control tube.
3. **Incubate Your Samples:** Incubate all tubes aerobically at 35 ± 2°C for 24 hours.
4. **Clean up:** Dispose of stock samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

→ Next Lab Session

1. **Obtain Your Samples:** Put on your lab safety gear, and then collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Examine Your Samples:** Examine the samples for color changes.
3. **Record Your Result:** Refer to Table 35.1 and Figure 35.2 when interpreting your results. Record your results in the table provided in the data sheet at the end of this exercise.
4. **Clean up:** Dispose of the samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 35

DATA SHEET

Urea Hydrolysis (Urease Test)

During the Lab

1 Using Table 35.1 and Figure 35.2 for guidance, enter your findings in the following table.

Organism	Color Result	(+/–)	Interpretation (include enzyme)
Control			

Lab Related Questions

1 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ The media was originally designed as an agar plate.

_____ A positive test result indicates ammonium was made.

_____ A negative test result indicates urea was not hydrolyzed.

_____ Test results should be viewed no later than 48 hours after incubation.

2 _____ is the enzyme that hydrolyzed urea.

3 Choose the true statement(s) about urea (select all that apply).

a. Urea is a virulence factor.
b. Urea can serve as a nitrogen source.
c. Urea is present at high levels in urine.
d. Urea is only found in urine.
e. Urea is a byproduct of amino acid catabolism.

4 Choose the true statement(s) about urease (select all that apply).

a. Urease is a virulence factor.
b. Urease is only made by pathogens.
c. Urease is a hydrolytic enzyme.
d. Urease acts extracellularly.
e. Urease produces urine.

5 Choose the true statement about urea hydrolysis test media.

a. Only microbes that are urease positive can grow in the media.
b. The pH indicator methyl red is present in the media to detect pH changes.
c. The media is rich in nutrients.
d. The media can detect the production of urea.
e. The media can differentiate *Proteus* species from *E. coli*.

6 How would observing the test results at 48 hours likely affect the experimental outcome?

a. You would be less likely to observe a positive test result.
b. You would be less likely to observe a negative test result.
c. You would be more likely to observe a false-negative test result.
d. You would be more likely to observe a false-positive test result.
e. You would be more likely to observe contamination.

Critical Thinking

1 You are working in a clinical microbiology lab, and immediately after you open a patient's urine sample, your colleague, who is sitting near you, declares, "Wow, if that isn't a urease-positive sample, then I don't know what is!" How could your colleague possibly know anything about the probable urease test result when you haven't even performed a urease test?

2 Your professor informs you that the test media was prepared without the pH indicator, but that you should still be able to make test result conclusions using tools in the lab. How would you proceed?

EXERCISE 36

Assessing Sulfur Reduction, Indole Production, and Motility (SIM Media and General Motility Agar)

After doing this exercise, you should be able to:

1. Describe how to use media to test for a bacterium's sulfur reduction ability, indole production, and motility.
2. State the differences between SIM (sulfur reduction, indole production, motility) media and general motility agar.
3. Describe how to perform and interpret sulfur reduction, indole production, and motility tests.
4. Discuss why assessing sulfur reduction, indole production, and motility is useful.
5. Test bacteria for sulfur reduction ability, indole production ability, and motility, and interpret the test results.

Background

In this exercise, you will explore how to assess a bacterium's ability to reduce sulfur, make indole, and move. Learning if a bacterial isolate has these features assists with bacterial species identification. It's worth noting that we can detect any one of these three capabilities using a variety of tests; however, here we will focus on using SIM media (a three-in-one test that assesses sulfur reduction, indole production from tryptophan, and motility) and general motility agar. Before we dive into how we perform the SIM and general motility tests, let's first walk through each of these three features.

1. **Sulfur Reduction:** Bacteria accomplish sulfur reduction to H_2S in two different ways, depending on the enzymes present. Both mechanisms produce hydrogen sulfide (H_2S) gas, which we can detect using SIM (sulfur reduction, indole production, motility) media.[1]
   - The enzyme **cysteine desulfurase** catalyzes the hydrolysis of the sulfur-containing amino acid cysteine to pyruvate, ammonia (NH_3), and hydrogen sulfide (H_2S) during putrefaction (Fig. 36.1). The resulting pyruvate may enter fermentation or respiration pathways for energy extraction.
   - In a totally unrelated way, the enzyme **thiosulfate reductase** catalyzes the reduction of sulfur (in the form of sulfate) to H_2S at the end of an anaerobic respiratory electron transport chain (Fig. 36.2).
2. **Indole Production:** Bacteria that make the enzyme **tryptophanase** can hydrolyze the amino acid tryptophan to pyruvate, ammonia (by deamination), and indole (Fig. 36.3). The cell may then extract energy from pyruvate by funneling it into fermentation reactions or cellular respiration. SIM media allows us to assess bacteria for indole production.[2]

[1] The triple sugar iron (TSI) test also reveals information about a bacterium's ability to perform sulfur reduction. We discuss that test in Exercise 37.

[2] Theoretically, any media containing enough tryptophan can be used to detect indole production. SIM is one such media, but tryptone broth is another common media for this application.

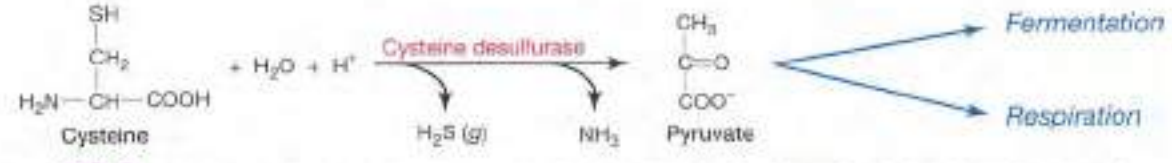


FIGURE 36.1 Sulfur Reduction: H_2S Production from Cysteine Hydrolysis (Cysteine Desulferase Reaction) • In putrefaction, cysteine desulfurase catalyzes the hydrolysis of the amino acid cysteine with the production of pyruvate, ammonia (NH_3), and hydrogen sulfide (H_2S).

$$3S_2O_3^{2-} + 4H^+ + 4e^- \xrightarrow{\text{Thiosulfate reductase}} 2SO_3^{2-} + 2H_2S\ (g)$$

FIGURE 36.2 Sulfur Reduction: H_2S Production from Anaerobic Respiration (Thiosulfate Reductase Reaction) • Anaerobic respiration with thiosulfate as the final electron acceptor produces hydrogen sulfide (H_2S).

3. **Motility:** Testing for motility will only reveal if a bacterium can move. This specific assessment does not reveal anything about the presence or absence of an enzyme or a biochemical product, and is therefore not technically considered a biochemical test. Some bacteria move using a single flagellum or multiple flagella; others use gliding or cilia. While we can note a bacterium's motility and occasionally its mechanism of motility by observing it under a microscope, here we review how to use SIM media and general motility agar to test if the bacterium can move. *Note:* Neither of these media reveals how the tested bacterium moves (e.g., flagella versus cilia).

Media Formulations and Test Result Interpretations

Let's now review how we can use media to assess sulfur reduction, indole production, and motility. Here we review SIM media *and* general motility agar. That said, your instructor may direct you to only use SIM media or they may include the general motility agar as an additional test to guard against inconclusive motility results that can occur when using SIM media. An inoculating needle is used to stab SIM and general motility agar.

Using SIM Media to Assess Sulfur Reduction

As previously reviewed, there are two main ways that bacteria reduce sulfur. In both cases, bacteria make hydrogen sulfide gas (H_2S). SIM media can detect H_2S production because the media contains iron in the form of ferrous ammonium sulfate. This ingredient combines with H_2S to form ferric sulfide (FeS), an easily visible black precipitate (Fig. 36.4). Therefore, any blackening of the SIM medium is an indication of sulfur reduction and a positive test. If there is no blackening in the SIM media, that indicates sulfur reduction did not occur, so the test result is negative (Fig. 36.5). Table 36.1 summarizes test results and interpretations for the "S" (sulfur reduction) part of SIM media.

FIGURE **36.4** **Indicator Reaction for H_2S Production in SIM Media** • Hydrogen sulfide (H_2S), a colorless gas, reacts with ferrous sulfate in SIM media to generate an easily detectable black precipitate.

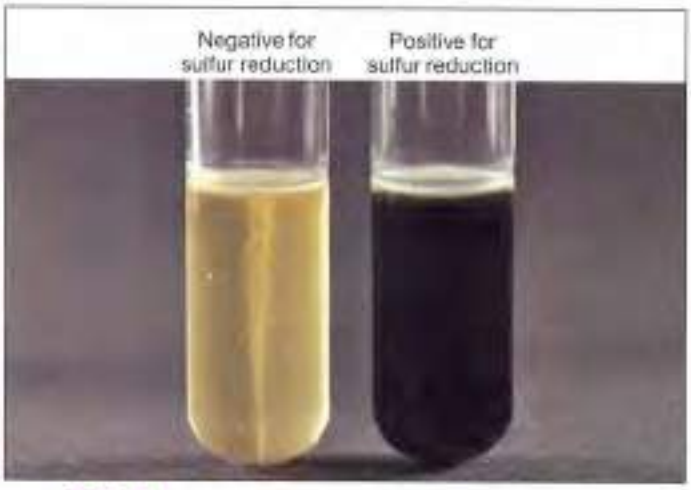


FIGURE **36.5** **Sulfur Reduction in SIM Media** • The lack of a black precipitate for the organism on the left indicates the bacterium did not make H_2S, this is a negative test result. The black precipitate, evident in the tube on the right, indicates the organism reduced sulfur and made H_2S, this is a positive test result.

TABLE **36.1** **Sulfur Reduction (H_2S Production) Test Results and Interpretations** •

Result	Interpretation	Symbol
No black in the medium	Sulfur is not reduced, or is not reduced in detectable amounts	−
Black in the medium	Sulfur reduction occurred (H_2S was made); organism either respires anaerobically with sulfate as the final electron acceptor (thiosulfate reductase) or hydrolyzes the amino acid cysteine (cysteine desulfurase)	+

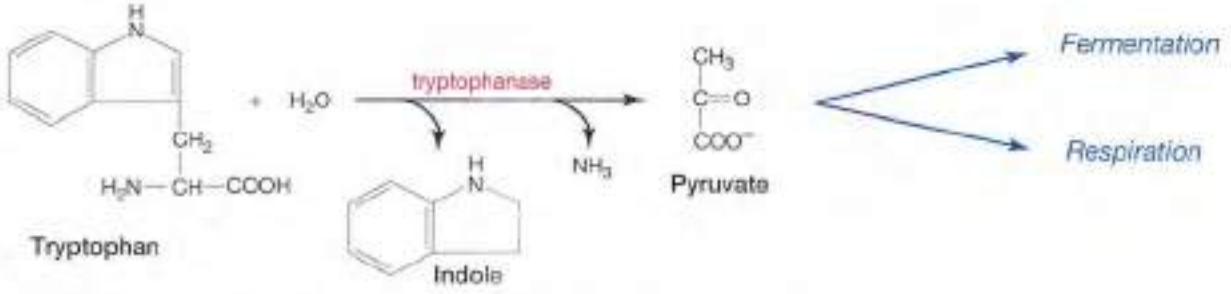


FIGURE **36.3** **Tryptophan Catabolism** • The enzyme tryptophanase breaks down the amine acid tryptophan.

Using SIM Media to Assess Indole Production

Tryptophan hydrolysis in SIM medium can be detected by the addition of Kovac's reagent after the incubation period. Kovac's reagent contains ***p*-dimethylaminobenzaldehyde** (DMABA) and HCl dissolved in amyl alcohol. When a few drops of Kovac's reagent are added to the tube, it forms a layer over the agar. DMABA reacts with any indole present and produces a quinoidal compound that turns the reagent layer red (Figs. 36.6 and 36.7). The formation of red color in the reagent layer indicates a positive reaction and the presence of tryptophanase. The test result is negative if a red color fails to develop after adding Kovac's reagent. Table 36.2 summarizes test results and interpretations for the "I" (indole production) part of SIM media.

TABLE **36.2** **Indole Production Test Results and Interpretations** •

Result	Interpretation	Symbol
No red layer evident after adding Kovac's reagent	Tryptophanase is absent and indole is not produced, or is produced but not in detectable amounts	–
Red layer evident after adding Kovac's reagent	Organism produces tryptophanase and hydrolyzes tryptophan into indole, NH_3, and pyruvate	+

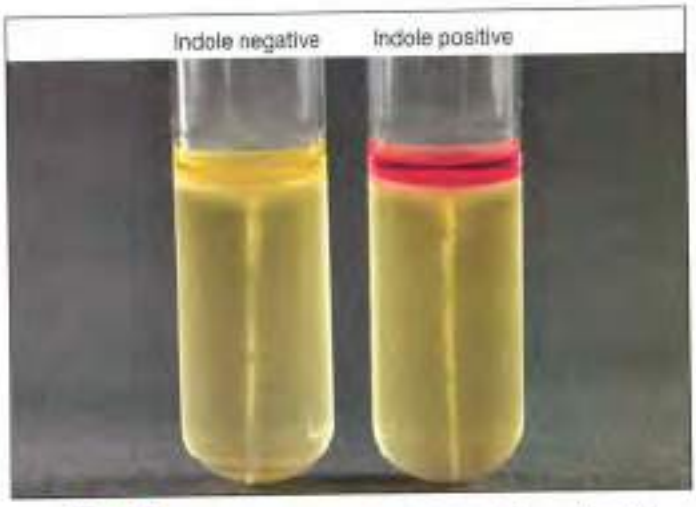


FIGURE **36.7** **Indole Production Test Results in SIM Media** • These SIM tubes were inoculated with an indole-negative organism on the left (no red layer evident after adding Kovac's reagent) and an indole-positive organism on the right (red layer evident upon adding Kovac's reagent). Notice how little Kovac's reagent is used.

Using SIM Media and General Motility Agar to Assess Motility

SIM agar is a semisolid medium that has a reduced agar concentration (0.35% vs. 1.5% in nutrient agar). Its semisolid formulation allows us to detect motility by looking to see if growth radiated away from the line of inoculation. Observing slightly fuzzy growth in *all* directions away from the line of inoculation is an indication of motility (Fig. 36.8). This should not be confused with the (seemingly) spreading growth produced by lateral movement of the inoculating needle when stabbing. Table 36.3 summarizes test results and interpretations for the "M" (motility) part of SIM media.

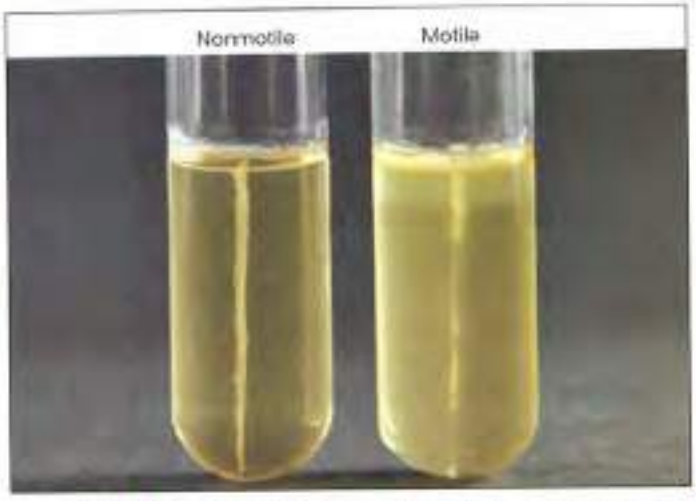


FIGURE **36.8** **Motility in SIM Medium** • Nonmotile bacteria will remain along the line of inoculation. In contrast, for motile bacteria, you should see a haziness in the agar radiating away from the line of inoculation. If results are not interpretable (vague or obscured by the black precipitate indicative of sulfur reduction), try general motility agar.

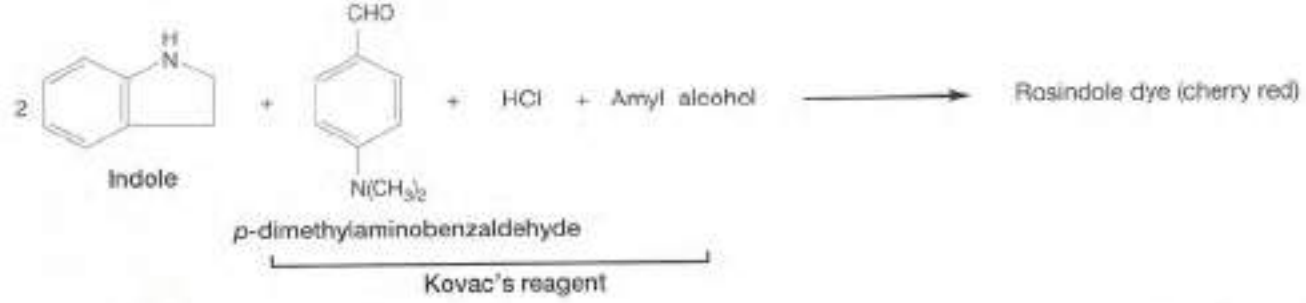


FIGURE **36.6** **Indicator Reaction for Indole Production in SIM Media** • Indole, if present, reacts with the DMABA in Kovac's reagent to produce a red color.

TABLE **36.3** Motility Test Results and Interpretations (SIM media) •

Result	Interpretation	Symbol
No growth radiating away from the original inoculation/ stab line	Nonmotile	–
Growth radiating outward from inoculation/stab line	Motile	+
Blackened medium (H_2S produced)	Motility not determined with this media (use general motility agar to assess motility)	ND

TABLE **36.4** General Motility Agar Test Results and Interpretations •

Result	Interpretation	Symbol
Red diffuse growth radiating outward from the stab line	Organism is motile	+
Red growth only along the stab line	Organism is nonmotile	–

General motility agar is another media that reveals information about motility. This agar is especially useful in cases where sulfur reduction obscures motility observations in SIM agar. Like SIM agar, general motility agar is a semisolid medium. Unlike SIM media, general motility agar only reveals information about a bacterium's motility.

As with SIM media, general motility agar is inoculated via a single stab with an inoculating needle. Following incubation, motile organisms are observed as a radiating growth that extends outward in all directions from the central stab line. A tetrazolium salt (TTC) is sometimes added to the medium to make interpretation easier. TTC is used by the bacteria as an electron acceptor. In its oxidized form, TTC is colorless and soluble; when reduced, it is red and insoluble (Fig. 36.9). As summarized in Table 36.4, a positive result for motility is indicated when the red (reduced) TTC is seen radiating outward from the central stab; a negative result shows red only along the stab line (Fig. 36.10).

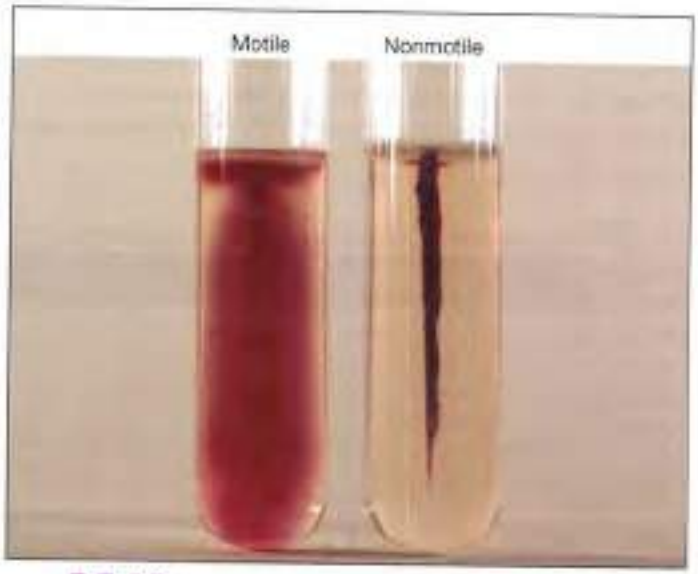
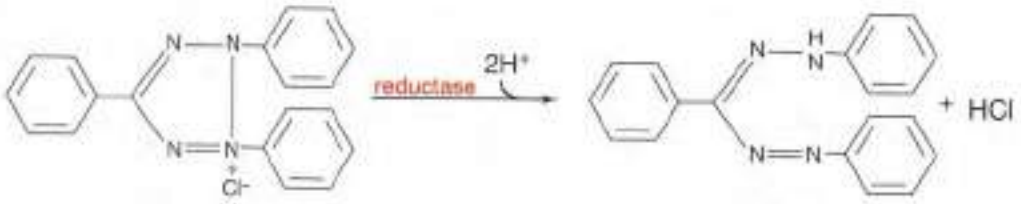


FIGURE **36.10** Results in General Motility Agar with TTC • A red color spreading away from the line of inoculation indicates the tested bacterium is motile. If the red color remains isolated to the line of inoculation, then the bacterium is nonmotile.



2,3,5-Triphenyltetrazolium chloride$_{oxidized}$ (TTC_{ox})
colorless and soluble

Formazan$_{reduced}$
red color and insoluble

FIGURE **36.9** General Motility Agar Indicator Reaction: Reduction of TTC • When 2,3,5-triphenyltetrazolium chloride (TTC) is reduced, it is converted to formazan, a red insoluble compound. In general motility media, this red compound serves as an indicator of bacterial growth location.

Clinical Applications

SIM medium is used to differentiate a variety of pathogenic bacteria. For example, sulfur-reducing members of *Enterobacteriaceae*, especially members of the genera *Salmonella*, *Shigella*, and *Proteus* are readily differentiated from H_2S negative *Morganella morganii* and *Providencia rettgeri*.

SIM medium also detects bacteria that can produce indole using the enzyme tryptophanase. The indole test is one component of the IMViC battery of tests (Indole, Methyl red, Voges-Proskauer, and Citrate) used to differentiate indole-positive *E. coli* from indole-negative members of the *Enterobacter*, *Klebsiella*, *Hafnia*, and *Serratia* genera.

In addition to the first two functions, SIM can assess bacterial motility, an important differential characteristic of *Enterobacteriaceae*, which are usually motile. Recall that the family *Enterobacteriaceae* includes the genera *Escherichia*, *Enterobacter*, *Klebsiella*, *Serratia*, and *Citrobacter* (all referred to as coliform bacilli) as well as *Proteus*. These genera encompass members of the normal microbiota of the intestinal tract, some of which act as true pathogens that cause a wide range of infections, while others are categorized as opportunistic pathogens. Aside from *Klebsiella* species, other *Enterobacteriaceae* coliforms are motile.

General motility agar is another media that reveals motility information. This media is especially useful if the motility results in SIM media are obscured by the black precipitate generated when H_2S is made.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Sulfur-indole-motility (SIM) media tubes (5; 4 to inoculate and 1 as a control)
- ❑ General motility agar tubes (5; 4 to inoculate and 1 as a control)
- ❑ Kovac's reagent
- ❑ Inoculating needles (wire or disposable)
- ❑ Microbial incinerator or Bunsen burner (if not using disposable inoculating needles)
- ❑ Fresh agar slant cultures of these recommended organisms:
  - ☐ *Escherichia coli*
  - ☐ *Micrococcus luteus*
  - ☐ *Proteus mirabilis* (BSL-2)[1]
  - ☐ *Streptococcus* species or *Staphylococcus epidermidis*

[1] *Note to instructor:* Approximately 75% of *Citrobacter freundii* strains are positive for sulfur reduction and it may serve as a suitable BSL-1 replacement for *P. mirabilis*. *Aeromonas hydrophila* may also work as a substitute.

Media and Reagent Recipes

Sulfur-Indole-Motility (SIM) Medium

Pancreatic digest of casein ($C_{38}H_{57}N_9O_9$)	20.0 g
Peptic digest of animal tissue	6.1 g
Ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)_2$)	0.2 g
Sodium thiosulfate ($Na_2S_2O_3$)	0.2 g
Agar	3.5 g
Distilled or deionized water	1.0 L

Adjust to pH 7.1–7.5 at 25°C

Kovac's Reagent

Amyl alcohol	75.0 mL
Hydrochloric acid, concentrated	25.0 mL
p-dimethylaminobenzaldehyde	5.0 g

General Motility Agar

Beef extract	3.0 g
Pancreatic digest of gelatin	10.0 g
Sodium chloride (NaCl)	5.0 g
Agar	4.0 g
Triphenyltetrazolium chloride (TTC)	0.05 g
Distilled or deionized water	1.0 L

Adjust to pH 7.1–7.5 at 25°C

PROCEDURE

6

You will use an inoculating needle (wire or disposable as directed by your instructor) to stab inoculate SIM medium with four organisms demonstrating a variety of results. If your instructor directs you to do so, you will also stab inoculate the same four bacteria onto general motility agar. When reading your SIM test results, make the motility and H_2S determinations before adding the Kovac's (indole) reagent. Wear a lab coat, gloves, and chemical eye protection when performing this procedure. ***Caution!*** **Follow BSL-2 precautions if using *P. mirabilis*.**

1. **Obtain Your Samples and Media:** Obtain the four bacterial stock cultures that you are going to test and five tubes of each media, SIM and (if directed) general motility agar. Four of the five tubes of each media will be inoculated; one of each media type will remain uninoculated. Label your tubes with the appropriate media name (e.g., SIM or motility), your name, the date, and the sample descriptor (e.g., organism name or uninoculated).

2. **Inoculate the Media:** Use an inoculating needle (disposable or wire) to stab inoculate four tubes of each media type (SIM and motility) with the provided test organisms. Insert the needle into the agar to within 1 cm of the bottom of the tube. Be careful to remove the needle along the original stab line so that you obtain a straight inoculation. Do not inoculate the control(s).
3. **Incubate Your Samples:** Incubate all tubes aerobically at 35±2°C for 24 to 48 hours.
4. **Clean up:** Save or dispose of stock samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

→ Next Lab Session

1. **Obtain Your Samples:** Put on your lab safety gear, and then collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Examine Your Samples for Sulfur Reduction and Motility:** Before you proceed to the indole test, examine the SIM media for sulfur reduction and motility. If you performed the general agar motility test, then also make your observations for those samples.
3. **Examine Your Samples for Indole Production:** Add Kovac's reagent to each SIM media tube (including the control) to a depth of 2–3 mm (*not mL*). After several minutes, observe for the formation of red color in the reagent layer.
4. **Record Your Result:** Refer to Tables 36.1, 36.2, and 36.3 along with Figures 36.5, 36.7, 37.8, and 36.10 for assistance with interpreting test results. Record your results in the tables provided in the data sheet at the end of this exercise.
5. **Clean up:** Dispose of the samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 36

DATA SHEET

Assessing Sulfur Reduction, Indole Production, and Motility (SIM Media and General Motility Agar)

During the Lab

1 Using Table 36.1 and Figure 36.5 as guides, record your results and interpretations for sulfur reduction in the following table.

Sulfur Reduction (H_2S Production)			
Organism	**Black PPT (Y/N)**	**(+/–)**	**Interpretation** (include enzyme)
Uninoculated control			

2 Using Table 36.2 and Figure 36.7 as guides, record your results and interpretations for indole production in the following table.

Indole Production			
Organism	**Red Color? (Y/N)**	**(+/–)**	**Interpretation** (include enzyme)
Uninoculated control			

3 Using Table 36.3 and Figures 36.8 and 36.10 as guides, record your results and interpretations for motility in the following tables.

Motility (SIM Media)			
Organism	Growth Location	(+/–)	Interpretation
Uninoculated control			

Motility (General Motility Agar)			
Organism	Result (location of red TTC)	Motility (+/–)	Interpretation

Lab Related Questions

1 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ General motility test agar can reveal if a bacterium uses flagella for motility.

_____ Thiosulfate reductase reduces H_2S.

_____ Sulfur reduction occurs during putrefaction.

_____ SIM media is an example of selective media.

2 The IMViC battery of tests includes:

a. Testing for motility.
b. Testing for indole production.
c. Testing for variable sulfur reduction.
d. Testing for constant sulfur reduction.
e. None of the above.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 36
DATA SHEET
(continued)

3 Choose the true statement(s) about sulfur reduction (select all that apply).

a. If sulfur is reduced, then a red layer is evident upon adding Kovac's reagent to SIM media.
b. Cysteine desulfurase performs sulfur reduction.
c. Sulfur reduction can help differentiate *Shigella* from *Salmonella*.
d. An organism that reduces sulfur will generate a fuzzy growth in SIM media.
e. Sulfur reduction can obscure motility test results in SIM agar.

4 Choose the true statement(s) about general motility agar (select all that apply).

a. This test is not technically a biochemical test.
b. The pH indicator methyl red is present in the media.
c. The test can differentiate members of the genera *Klebsiella* from other coliform genera.
d. The test is performed using semisolid agar.
e. 2,3,5-triphenyltetrazolium chloride serves as a color indicator in this test.

5 Choose the true statement(s) about the indole production test (select all that apply).

a. The test will be positive if the bacterium produces thiosulfate reductase.
b. The test is usually developed before 24 hours of incubation.
c. 2,3,5-triphenyltetrazolium chloride serves as a color indicator in this test.
d. Indole is made as a byproduct of tryptophan catabolism.
e. Kovac's reagent is added to detect indole production.

6 The sulfur reduction test is not able to differentiate H_2S produced by anaerobic respiration and H_2S produced by putrefaction. This inability is the result of:

a. Limited test sensitivity.
b. Limited test specificity.
c. Limited activity of thiosulfate reductase.
d. Limited bacterial growth on SIM agar.
e. Limited activity of cysteine desulfurase.

Critical Thinking

1 Consider the TTC indicator in the general motility agar. Why is it essential that the reduced TTC be insoluble?

2 Which ingredient(s) could be eliminated if SIM media were strictly used for testing indole production? Explain.

— Lab Notes —

EXERCISE 37

Triple Sugar Iron Agar (TSIA)/Kligler Iron Agar (KIA)[1]

After doing this exercise, you should be able to:

1. Describe how to use TSIA and/or KIA to test for a bacterium's sulfur reduction ability.
2. Describe how TSIA and/or KIA help us determine a bacterium's ability to ferment glucose, lactose, and/or sucrose.
3. State the differences between TSIA and KIA.
4. Describe how to perform and interpret TSIA and KIA tests.
5. Discuss why assessing fermentation and H_2S production properties is useful.
6. Perform TSIA and/or KIA tests and interpret the results.

Background

This exercise is performed using either triple sugar iron agar (TSIA) or Kligler iron agar (KIA). Each is an enriched medium that differentiates bacteria (especially enterics, *Enterobacteriaceae*) based on sulfur reduction and sugar fermentation. Leading up to this exercise, we reviewed pathways for carbohydrate fermentation (Ex. 27) and hydrogen sulfide (H_2S) production (Ex. 36). If you wish to review those biochemical processes in more detail, refer to those exercises. However, as a basic reminder, recall that hydrogen sulfide (H_2S) is a common byproduct of sulfur reduction pathways, and acids are common byproducts of carbohydrate fermentation. TSIA and KIA tests rely on detecting these key pathway products (H_2S and acids) to test for fermentation and sulfur reduction in one inexpensive and easily performed test.

Media Formulations

As the name implies, TSIA or *triple* sugar iron agar contains three sugars: lactose, sucrose, and glucose. It also contains animal proteins as carbon and nitrogen sources and sodium thiosulfate as an oxidized sulfur source. Phenol red serves as the pH indicator; it appears yellow at pH less than 6.8 and reddish above pH 7.4, so it readily indicates if acidic fermentation byproducts are present. Ferrous sulfate allows us to determine if the tested bacteria reduce sulfur to make H_2S; if they do, then the generated H_2S reacts with ferrous sulfate to form a black precipitate. KIA is almost the same formulation as TSIA, the difference is that KIA contains two sugars (lactose and glucose) versus the three sugars in TSIA.

Both TSIA and KIA are prepared as a shallow agar slant with a deep butt. This format provides both aerobic and anaerobic growth environments (we'll review the implications of this in the next section). Both media are double inoculated, first by a stab in the agar butt and then by a fishtail streak of the slant.

There are two incubation windows for these tests: one time frame is for detecting carbohydrate fermentation and the second is for assessing H_2S production. Cultures are incubated for 18–24 hours before interpreting carbohydrate fermentation. After interpreting the carbohydrate fermentation results, samples are re-incubated for an additional 24 hours before making a final H_2S determination.

Test Interpretations

There are two environmental situations established in the medium, an aerobic environment exists in the slant whereas the butt of the tube provides an anaerobic environment. This difference in oxygen levels, combined with differences in sugar concentrations (10 times as much lactose and sucrose as glucose in the medium), facilitates a differential coloration of the slant and/or butt based on what sugar(s) the bacterium ferments. Many reactions in various combinations are possible. Table 37.1 summarizes the possible color changes you may observe and what each observation indicates in terms of fermentation reactions that occurred and/or H_2S production.

- **Yellow Butt and Red Slant = Glucose Fermentation Only:** When glucose-only fermenters grow on TSIA or KIA, acidic products lower the pH and turn the entire medium yellow within just a few hours. However, if you were to recheck your sample at the recommended 18- to 24-hour mark, you'd see that *only* the butt of the tube was yellow. This phenomenon of the medium's slant changing from red (the original color at inoculation) to yellow (the color after a few hours of incubation) and then *back* to red (the final color at 18–24 hours) is called **reversion.**

[1] This exercise is written for either triple sugar iron agar (TSIA) or Kligler iron agar (KIA) use. In form and function, the two media are virtually identical except KIA includes two sugars versus TSIA's three sugars. The results for TSIA and KIA are read the same way. In results indicating fermentation of more than one carbohydrate, TSIA is reported as *glucose and lactose and/or sucrose fermentation*; KIA (because it contains no sucrose) is reported as *glucose and lactose fermentation*.

The reversion process only occurs in the slant because bacteria metabolize glucose under the aerobic conditions of the slant much faster than they metabolize glucose under the anaerobic conditions deeper in the medium. And because the medium has a low glucose concentration (0.1%), bacteria metabolize most (if not all) of the glucose in the slant within about 12 hours. As the glucose in the slant diminishes, bacteria growing in that region switch to metabolizing proteins in the medium as an energy source. As the bacteria break down amino acids (the building blocks of proteins), ammonia is made. Ammonia is a base, so as it accumulates, it alkalizes the slant (pH increases), restoring the slant to its *original* red color.

In contrast, bacteria grow slowly in the anaerobic environment at the butt of the tube and therefore they do not burn through the glucose as fast as bacteria growing in the aerobic slant environment. Because bacteria growing deep in the medium have plenty of glucose to meet their growth needs, they will not switch to metabolizing proteins for energy. This means bacteria in the butt of the tube will not produce ammonia, so the butt remains acidic and therefore remains yellow. Thus, if your TSIA or KIA sample has a red slant and yellow butt at the 18- to 24-hour mark, then the tested bacteria ferments glucose, but not lactose and/or sucrose (see the tube on the far left of Fig. 37.1). If the bacteria make gas during fermentation, you may see fissures in the medium or the agar may lift slightly off the bottom of the tube (see the tube on the far right of Fig. 37.1).

- **Yellow Slant and Yellow Butt = Glucose *and* Lactose *and/or* Sucrose Fermentation:** Organisms that ferment glucose *and* lactose *and/or* sucrose turn TSIA and KIA yellow throughout. (Refer to Fig. 27.1 for the biochemical relationship between these three fermentations.) Because other sugars in the medium (lactose and sucrose for TSIA/lactose for KIA) are 10 times more concentrated than glucose, these sugars remain plentiful in the slant even after glucose is depleted. Therefore, the bacteria do not need to resort to protein catabolism to survive and can instead continue to ferment sugars throughout the 18- to 24-hour incubation period. This means the slant and butt will remain yellow (Fig. 37.1, far right tube).

  As such, a TSIA sample with a yellow slant and butt at the 18- to 24-hour mark indicates the inoculated organism ferments glucose and at least one (though possibly *both*) of the other two sugars. That is, glucose fermented *and* lactose and/or sucrose also fermented.

For KIA, because there are only two sugars present (glucose and lactose), if the slant and butt are both yellow, you can deduce that both sugars fermented. As described in the previous section, if the bacteria make gas as a byproduct of fermenting any of the sugars, the medium may exhibit fissures or the agar may slightly lift from the bottom of the tube.

- **Red Slant and Red Butt = No Fermentation:** An organism that does not ferment any of the carbohydrates but utilizes peptone and amino acids will alkalinize the medium and turn it red (usually a brighter red than uninoculated medium). If the organism uses the peptone aerobically *and* anaerobically, both the slant and butt will appear a bright red. An obligate aerobe will turn only the slant bright red. The difference between a red butt and a butt unchanged by the organism may be subtle; therefore, comparison with an uninoculated control is always recommended.
- **Black Coloration = H_2S Production (Sulfur Reduction):** Bacteria that reduce thiosulfate present in the medium or break down the amino acid cysteine in the medium's peptone will make H_2S (Figs. 36.1 and 36.2). Ferrous sulfate reacts with any generated H_2S to form a black precipitate that, when formed, is easily detectable in the butt of the tube. Acidic conditions must exist for thiosulfate reduction. Therefore, black precipitate in the medium is an

FIGURE **37.1** **Results in Kligler Iron Agar Slants** • These KIA slants from left to right illustrate: alkaline slant/acid butt (K/A); alkaline slant/no change in the butt (K/NC); uninoculated control; alkaline slant/acid butt, hydrogen sulfide present (K/A, H_2S); and acid slant/acid butt, gas (A/A, G). TSIA tubes with the same appearances would be scored the same. Refer to Table 37.1 for interpretations.

indication of sulfur reduction *and* at the very least, glucose fermentation. If the black precipitate obscures the color of the butt, you can reasonably assume the organism fermented glucose. However, you must still note the color of the slant to determine if any other sugars were fermented.

A Note on Test Result Observation Timing

Because we are observing a delicate balance between slant versus butt acidification or alkalinization, timing is critical when reading TSIA and KIA results. An early reading could reveal yellow throughout the medium, leading you to believe that the organism ferments more than one sugar when it simply has not yet exhausted the glucose. A reading after all the sugars have been depleted could reveal a yellow butt and red slant, leading you to falsely believe that the organism is a glucose-only fermenter. Interpreting the H_2S production results too early could lead you to wrongly conclude the organism is unable to form H_2S.

TABLE **37.1** TSIA and KIA Test Results and Interpretations •

Result	Interpretation	Symbol
Yellow slant/yellow butt—KIA	Glucose and lactose fermentation with acid accumulation in slant and butt	A/A
Yellow slant/yellow butt—TSIA	Glucose and lactose and/or sucrose fermentation with acid accumulation in slant and butt	A/A
Red slant/yellow butt	Glucose fermentation with acid production; proteins catabolized aerobically (in the slant) with alkaline products (reversion)	K/A
Red slant/red butt	No fermentation; peptone catabolized aerobically and anaerobically with alkaline products; isolate is not from *Enterobacteriaceae*	K/K
Red slant/no change in the butt	No fermentation; peptone catabolized aerobically with alkaline products; isolate is not from *Enterobacteriaceae*	K/NC
No change in slant/no change in butt	Organism is growing slowly or not at all; isolate is not from *Enterobacteriaceae*	NC/NC
Black precipitate in the agar	Sulfur reduction (an acid condition from glucose fermentation exists in the butt, even if the yellow color is obscured by the black precipitate)	H_2S
Cracks in or lifting of agar	Gas production	G

Clinical Applications

Recall that the family *Enterobacteriaceae* is comprised of rod-shaped, Gram-negative bacteria that includes harmless bacteria as well as familiar pathogens (e.g., *Escherichia coli*, *Salmonella*, and others). TSIA and KIA are primarily used to differentiate members of *Enterobacteriaceae* based on sugar fermentation profiles and H_2S production. Many of the bacteria that can ferment sugars in the butt of TSIA and KIA are enterobacteria, so these tests can also help us distinguish *Enterobacteriaceae* from other Gram-negative rods such as *Pseudomonas aeruginosa*, which does not ferment sugars in the butt of the tube.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Triple sugar iron agar (TSIA) and/or Kligler iron agar (KIA) slants (5)
- ❑ Wire inoculating needle and loop or disposable inoculating needle and loop
- ❑ Microbial incinerator or Bunsen burner (if not using disposable inoculating needles)
- ❑ Fresh agar slant cultures of these recommended organisms[2]:
  - ☐ *Escherichia coli*
  - ☐ *Morganella morganii* (BSL-2)
  - ☐ *Pseudomonas aeruginosa* (BSL-2)
  - ☐ *Salmonella typhimurium* (BSL-2)
  - ☐ *Streptococcus* species or *Staphylococcus epidermidis*

[2] ***Note to instructor:*** If your lab is not equipped to handle BSL-2 organisms, these will be adequate substitutes for the recommended BSL-2 organisms: *Alcaligenes faecalis*, *Citrobacter freundii* (Hardy Diagnostics #0315P), and *Citrobacter koseri* (Hardy Diagnostics #0106P).

Medium Recipes

Triple Sugar Iron Agar (TSIA)

Pancreatic digest of casein ($C_{38}H_{57}N_9O_9$)	10.0 g
Peptic digest of animal tissue	10.0 g
Dextrose (glucose) ($C_6H_{12}O_6$)	1.0 g
Lactose ($C_{12}H_{22}O_{11}$)	10.0 g
Sucrose ($C_{12}H_{22}O_{11}$)	10.0 g
Ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)_2$)	0.2 g
Sodium chloride (NaCl)	5.0 g
Sodium thiosulfate ($Na_2S_2O_3$)	0.2 g
Agar	13.0 g
Phenol red	0.025 g
Distilled or deionized water	1.0 L

Adjust to pH 7.1–7.5 at 25°C

Kligler Iron Agar (KIA)

Pancreatic digest of casein ($C_{38}H_{57}N_9O_9$)	10.0 g
Peptic digest of animal tissue	10.0 g
Lactose ($C_{12}H_{22}O_{11}$)	10.0 g
Dextrose (glucose) ($C_6H_{12}O_6$)	1.0 g
Ferric ammonium citrate ($C_6H_5O_7 \cdot Fe \cdot NH_3$)	0.5 g
Sodium chloride (NaCl)	5.0 g
Sodium thiosulfate ($Na_2S_2O_3$)	0.5 g
Agar	15.0 g
Phenol red	0.025 g
Distilled or deionized water	1.0 L

Adjust to pH 7.2–7.6 at 25°C

PROCEDURE

Today, you will inoculate four TSIA or KIA slants. Use a large inoculum and try not to introduce excessive air when stabbing the agar butt. All tubes should be removed from the incubator after no more than 24 hours. Wear a lab coat, gloves, and chemical eye protection when performing this procedure. ***Caution!*** If you are studying any BSL-2 organisms, follow BSL-2 precautions.

1. **Obtain Your Samples and Media:** Obtain five slants and the sample bacteria that you will inoculate onto the media. Label four of the slants with the names of the organisms, your name, and the date. Label the fifth slant "control."
2. **Inoculate the Media:** Heavily inoculate four slants with the test organisms using an inoculating needle (not a loop). First stab the agar butt, and then streak the slant. Do not inoculate the control. Be sure to properly dispose of the inoculating needle (if a disposable one is used). Otherwise, incinerate the inoculating needle.
3. **Incubate Your Samples:** Incubate all tubes aerobically at 35±2°C for 18–24 hours (no more or less). Be sure the caps are loose enough to allow gas exchange.
4. **Clean up:** Save or dispose of stock samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

→ Next Lab Session

1. **Obtain Your Samples:** Put on your lab safety gear, and then collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Examine Your Samples:** Examine the tubes for characteristic color changes and gas production. The proper format for recording results is: slant reaction/butt reaction, gas production, hydrogen sulfide production. For example, a tube showing yellow slant, yellow butt, fissures in or lifting of the agar, and black precipitate would be recorded as: A/A, G, H_2S.
3. **Record Your Result:** Using Table 37.1 as a guide, record your results in the tables provided in the data sheet at the end of this exercise.
4. **Clean up:** Dispose of the samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 37

DATA SHEET

Triple Sugar Iron Agar (TSIA)/ Kligler Iron Agar (KIA)

During the Lab

1 Using Table 37.1 and Figure 37.1 as guides, record your results and interpretations in the following table.

Organism	Color and Gas Result	Symbol	Interpretation (include enzyme when known)
Control			

Lab Related Questions

1 Assume that after 18 hours of incubation your sample has a black precipitate that obscures your view of the underlying butt color. What is the best course of action?

a. Repeat the test and observe the results at 15 hours.
b. Assume the butt was alkalinized and would have appeared red.
c. Assume the butt was alkalinized and would have appeared yellow.
d. Assume the butt was acidified and would have appeared red.
e. Assume the butt was acidified and would have appeared yellow.

2 Choose the true statement(s) (select all that apply).

a. TSIA is a selective media.
b. KIA does not reveal information about sucrose fermentation.
c. There is more glucose present in TSIA than in KIA.
d. KIA must be incubated longer than TSIA.
e. Sulfur reduction can obscure fermentation results in TSIA, but not KIA.

3 When interpreting the TSIA test, if we observe a yellow slant, then we use the phrasing that lactose and/or sucrose was fermented. Why is this done?

a. Because a test limitation is that we can't know if one or both sugars were fermented.
b. Because if sucrose was fermented, it is likely that lactose was fermented first.
c. Because if lactose was fermented, it is likely that sucrose was as well.
d. Because the media may contain either sucrose or lactose.
e. Because sucrose is derived from lactose fermentation.

4 What is the role of the ferrous sulfate in the media?

a. It selects for the growth of enteric bacteria.
b. It promotes anaerobic fermentation reactions.
c. It serves as an indicator of H_2S production.
d. It serves as a pH indicator.
e. It is added as a nutrient.

5 The term *reversion* as used in the TSIA and KIA tests best describes:

a. The switching of metabolism from lactose fermentation to sucrose fermentation.
b. The switching of metabolism from sucrose fermentation to glucose fermentation.
c. A change in the butt color from red to yellow.
d. A change in the slant color from yellow to red.
e. A change in the slant color from red to yellow.

6 Under what condition(s) may you observe a red butt and red slant of a TSIA tube after 18–24 hours of incubation? (Select all that apply.)

a. Glucose levels were so high in the media that the tested bacterium couldn't ferment it all in 18–24 hours.
b. The organism failed to ferment glucose.
c. The organism fermented lactose, but not sucrose.
d. The pH indictor was not added to the media.
e. The organism failed to ferment lactose and sucrose.

Critical Thinking

1 How would the test results be affected if TSIA medium was prepared with the same concentration of lactose and sucrose as glucose, but the test was still observed at 18–24 hours?

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 37
DATA SHEET
(continued)

2 Your lab partner obtained the following results for TSIA: A/A.

You obtained the following results for KIA: K/A.

Assuming you used the same test organism, what best explains the difference in observed results?

— Lab Notes —

EXERCISE 38

Coagulase Test

After doing this exercise, you should be able to:

1. Describe how the coagulase test is performed.
2. Explain what the coagulase test reveals about a bacterial species.
3. State the applications of the coagulase test.
4. Perform a coagulase test and interpret the test results.

Background

This test detects if bacteria can make **coagulase**, an enzyme that most pathogenic Staphyococcus aureus make. There are two forms of coagulase: **free coagulase** and **bound coagulase** (also called **clumping factor**). Free coagulase is secreted (released from the cell) as an extracellular enzyme whereas clumping factor is attached to the bacterial cell wall.

Upon release into the extracellular environment, free coagulase reacts with **coagulase-reacting factor** (**CRF**), a protein found in plasma (the liquid portion of blood). This interaction triggers a process that resembles the normal blood clot formation mechanism, namely, the soluble protein fibrinogen is converted into an insoluble and highly polymerizable protein, fibrin. As fibrin proteins interact, they form a mesh-like protein network. Normally, this fibrous network would serve as a blood clot scaffold, capturing platelets and red blood cells to stifle bleeding.

However, in this case, the fibrous protein barrier shields the coagulase-producing bacteria from host immune system protections (e.g., elimination by phagocytic white blood cells) and it also promotes biofilm production[1], a situation associated with recurring infections that can be challenging to eradicate due to decreased drug susceptibility. Consequently, coagulase-positive *Staphylococcus aureus* tends to be more virulent (more likely to cause severe disease) than coagulase-negative *Staphylococcus* species.

Bound coagulase generates the same protective fibrin protein barrier as free coagulase, but the mechanisms of barrier generation differ. In contrast to free coagulase, bound coagulase *directly* converts fibrinogen to fibrin without CRF. When bound coagulase reacts with plasma fibrinogen, the precipitated fibrin links the cells to produce visible clumps.

[1] A biofilm is a microbial community of cells attached to a surface via a protective matrix.

Performing and Interpreting the Tube Coagulase Test

There are two forms of the coagulase test: the tube test and the slide test. The tube test detects the presence of either bound or free coagulase whereas the slide test only detects bound coagulase. Both tests utilize rabbit plasma treated with anticoagulant to help reduce the likelihood of false-positive results. The most common anticoagulant added to coagulase tube media is ethylenediaminetetraacetic acid (EDTA), which binds calcium ions thereby interrupting blood clotting unless coagulase is present. In this lab exercise we will only perform the tube test.

To perform a tube coagulase test, we inoculate rabbit plasma with a pure culture of the test bacterium. Plasma coagulation (including any thickening or formation of fibrin threads) within 24 hours is a positive reaction (Fig. 38.1). During the first 4 hours of incubation, it is common practice to periodically observe the inoculated tubes for clotting. If clotting does not occur within the first 4 hours, then samples undergo an overnight incubation, but incubation should never exceed a total of 24 hours. Adhering to the maximum 24-hour incubation is important because after 24 hours, staphylococcal fibrinolytic enzymes may dissolve the fibrin network leading to a false-negative result. Table 38.1 summarizes how to interpret tube coagulase results.

FIGURE **38.1** **Coagulase Tube Test Results in Rabbit Plasma** ■ Note the liquid nature of the coagulase-negative sample versus the media solidification seen in the case of coagulase-positive bacteria.

TABLE **38.1** Coagulase Tube Test Results and Interpretations •

Result	Interpretation	Symbol
Medium solidified within 24 hours or there is evident clumping/coagulation (even if the media did not entirely solidify).	Free or bound coagulase present; plasma coagulated; probable *Staphylococcus aureus*	+
Medium remains liquid after 24 hours; no signs of coagulation are evident	Plasma did not coagulate; coagulase was not detectable; unlikely to be *S. aureus*	–

Clinical Applications

The coagulase test differentiates coagulase-positive *Staphylococcus aureus* from other usually less virulent coagulase-negative *Staphylococcus* species.

Staphylococcus species such as *S. aureus, S. epidermidis,* and *S. saprophyticus* are common members of the normal human microbiome (the collection of microbes that reside in and on humans). These species are most prevalent on the skin and in the upper respiratory tract (especially the nose). When these bacteria remain in their normal microbiome niches, they are not usually problematic. However, if they invade the body (often, though not exclusively, via a skin wound), they can cause disease.

S. aureus causes infections ranging from superficial skin infections like impetigo to deeper infections such as cellulitis (deep skin infection), osteomyelitis (bone infection), and bacteremia (blood infection). Certain *S. aureus* strains also make specialized toxins that lead to toxin-mediated syndromes such as scalded skin syndrome, toxic shock syndrome, and staphylococcal food poisoning.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Sterile rabbit plasma tubes with EDTA (4) (0.5 mL in 12 mm × 75 mm test tubes)
- ❑ Sterile 1-mL transfer pipettes (2)
- ❑ Sterile wooden applicators or disposable plastic loops (6)
- ❑ Sterile saline (0.9% NaCl)
- ❑ Microscope slides (2)
- ❑ Fresh agar slant cultures of these recommended organisms:
  - ☐ *Staphylococcus aureus* (BSL-2)
  - ☐ *Staphylococcus epidermidis*

PROCEDURE

You will perform the coagulase tube test. Read the tube test no later than the following day. It would be most efficient if you inoculate the tube tests as early in your lab period as possible, with the hope that you may observe a positive result before class is over. Wear a lab coat, gloves, and chemical eye protection when performing this procedure. ***Caution!*** Follow BSL-2 precautions throughout this lab.

1. **Obtain Your Samples:** Obtain three coagulase tubes. Label two tubes with the names of the organisms, your group name, and the date. Label the third tube "control."
2. **Inoculate the Rabbit Plasma:** Using a sterile wooden applicator or disposable loop, inoculate two tubes of rabbit plasma with the test organisms. There should be slightly visible turbidity. Dispose of the stick/loop immediately and properly. Then mix the contents by gently rolling the tube between your hands. Do not inoculate the control.
3. **Incubate the Samples:** Incubate all tubes at 35±2°C for up to 24 hours, checking for coagulation periodically for the first 4 hours or remainder of your lab. Do not exceed 24 hours of incubation. If you do not meet within the next 24 hours, the samples should be removed to a refrigerator until your next lab period.
4. **Clean up:** Dispose of the materials as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

→ Next Lab Session

1. **Obtain Your Samples:** Remove all tubes from the incubator no later than 24 hours after inoculation.
2. **Examine the Samples:** Examine each tube for clotting of the plasma and record your results on the data sheet at the end of this exercise. Refer to Figure 38.1 and Table 38.1 when making your interpretations.
3. **Clean up:** Dispose of the materials as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 38

DATA SHEET

Coagulase Test

During the Lab

1 Refer to Table 38.1 when recording and interpreting your results in the following table.

Tube Test Results		
Organism	**Result**	**Interpretation (include coagulase type and provisional identification)**
Uninoculated control		

Lab Related Questions

1 Why is EDTA included in the medium?

a. It promotes coagulation if coagulase is present.
b. It inhibits coagulation if coagulase is present.
c. It promotes coagulation if coagulase is absent.
d. It inhibits coagulation if coagulase is absent.
e. It serves as an activator of the enzyme coagulase.

2 Which of the following would you expect to make coagulase?

a. All *Staphylococcus* species
b. *Staphylococcus aureus*
c. *Staphylococcus epidermidis*
d. *Staphylococcus saprophyticus*
e. None of the above

3 Choose the false statement(s) about free coagulase (select all that apply).

a. It generates a fibrin matrix by indirectly triggering fibrin polymerization.
b. It generates a fibrin matrix by directly polymerizing fibrin.
c. It is a secreted extracellular enzyme.
d. It is not associated with increased virulence.
e. It is only detectable via the slide coagulase test.

4 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ Clumping factor is also known as free coagulase.

_____ Bacteria that make coagulase are typically less virulent than bacteria that lack coagulase.

_____ Coagulase may help bacteria form biofilms.

_____ Bacteria that make coagulase should liquify the test medium within 24 hours.

5 If an organism does not have catalase then (select all that apply):

a. It is not able to cause disease.
b. It will not solidify the test medium.
c. It is likely to be *S. aureus*.
d. It is unlikely to be *S. aureus*.
e. It is unlikely to be found on the skin.

Critical Thinking

1 You and your lab partner each inoculated the rabbit serum media with the same test bacterium. However, their sample appears to manifest clumping by 2 hours whereas yours does not manifest clumping until the 4-hour mark. What is the most likely explanation for this observed time difference?

2 A negative coagulase test, even if performed correctly, does not rule out the presence of *S. aureus*. Why?

EXERCISE 39

Gelatin Hydrolysis (Gelatinase Test)

After doing this exercise, you should be able to:

1. Explain what the gelatinase test reveals about a bacterial species.
2. Describe how to perform and interpret the gelatin hydrolysis test.
3. State the applications of the gelatin hydrolysis test.
4. Perform a gelatin hydrolysis test and interpret the test results.

Background

The gelatin hydrolysis test (or gelatinase test) reveals if a bacterium makes extracellular enzymes called **gelatinases** that can hydrolyze the protein gelatin. Gelatinases are often virulence factors because they can help certain bacteria invade deeper tissues in animals. Gelatin, which is derived from collagen (a fibrous protein abundant in animal connective tissues), is hydrolyzed in two stages (Fig. 39.1). First, gelatinases break down the larger gelatin protein into polypeptides—shorter stretches of amino acids. Next, the polypeptides are further hydrolyzed to release individual amino acids. Bacteria may then import the individual amino acids and further metabolize them as nutrients or use them as building blocks to make proteins.

FIGURE **39.1** **Gelatin Hydrolysis** • Gelatinases hydrolyze gelatin to release amino acids.

FIGURE **39.2** **Gelatin Hydrolysis Test Results** • Slightly tilting the sample tube reveals if the media is solid or liquid. Before making your observations, confirm that the uninoculated control's media is solid. If it is not, then refrigerate any liquid samples and the uninoculated control until the media for the control is solidified. Gelatinase-positive bacteria will liquefy the media (**top**). Gelatinase-negative bacteria do not liquefy the media (**bottom**).

Gelatin Medium Formulation and Test Result Interpretation

To test for gelatinase, a pure culture of the test bacterium is inoculated into a tube of nutrient gelatin, a test medium composed of gelatin, peptone, and beef extract. Nutrient gelatin differs from most other solid media in that the solidifying agent (gelatin) is also the substrate for enzymatic activity. Consequently, when a tube of nutrient gelatin is stab inoculated with a gelatinase-positive organism, secreted gelatinase (or gelatinases) will liquefy the medium. Gelatinase-negative organisms do not secrete gelatinases; therefore, the medium will remain solid (Figs. 39.2 and 39.3).

A slight disadvantage of nutrient gelatin is that it liquifies at 28°C (82°F). Therefore, inoculated tubes are typically incubated at 25°C (77°F) along with an uninoculated control to verify that any resulting liquefaction is not temperature related. At this lower incubation temperature, a 7-day incubation period is usually sufficient to see liquefaction of the medium. However, gelatinase activity is very slow in some organisms. All tubes still negative after 7 days should be incubated an additional 7 days.

If time is of the essence, you can incubate the samples at 37°C for 2–7 days. However, because this higher temperature will liquify the gelatin medium, an

FIGURE **39.3** **Crateriform Liquefaction** • This form of liquefaction also may be of diagnostic use because not all gelatinase-positive microbes liquefy the gelatin completely. Shown here is an organism liquefying the gelatin in the shape of a crater (**arrow**).

uninoculated control must be incubated along with inoculated samples and before making observations, *all* samples must be refrigerated/iced until the uninoculated control solidifies. *Note:* Gelatinases usually act at the medium's surface, so shaking the tube while it is warm can generate a false-negative result. Use Table 39.1 to help you interpret your test results.

TABLE 39.1 Gelatin Hydrolysis Test Results and Interpretations

Result	Interpretation	Symbol
Gelatin is liquid (control is solid)	Gelatinase is present.	+
Gelatin is solid	Gelatinase is absent.	–

Clinical Applications

Gelatinases help certain bacteria invade host tissues to establish infection; therefore, these enzymes are important virulence factors for certain pathogens. The gelatin hydrolysis test can help us differentiate *Staphylococcus aureus*, which is gelatinase-positive, from gelatinase-negative *S. epidermidis*. It also helps us to distinguish *Serratia* and *Proteus* species—gelatinase-positive members of *Enterobacteriaceae*—from others in the family, which are usually gelatinase negative. *Bacillus*, *Clostridium*, and *Clostridioides* species are often gelatinase positive, so this test can help identify important pathogens like *B. anthracis*, *B. cereus*, *C. tetani*, *C. perfringens*, and *C. difficile*.

6

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Inoculating needles (wire or disposable)
- ❑ Tubes of solidified nutrient gelatin (3)
- ❑ Ice bath or refrigerator
- ❑ Fresh cultures of:
  - ❑ *Bacillus subtilis* or *B. cereus*
  - ❑ *Escherichia coli*

Medium Recipe

Nutrient Gelatin

Beef extract	3.0 g
Peptone	5.0 g
Gelatin	120.0 g
Distilled or deionized water	1.0 L

Adjust to pH 6.6–7.0 at 25°C

PROCEDURE

You will inoculate two nutrient gelatin tubes and incubate them for up to a week. Your instructor may have you incubate samples at 25°C or warmer, depending upon the time constraints for your course and their experiential preferences. To determine if media liquefaction is due to gelatinase activity or a temperature increase, you must include an uninoculated control among the samples. If the media of the uninoculated control is liquid after incubation, then refrigerate the liquified samples until the control is solidified. The control will usually solidify within 15–20 minutes of refrigeration. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

1. **Obtain and Label the Media:** Obtain three tubes of nutrient gelatin. Label two tubes with the name of the organisms that you'll inoculate into each tube, your name, and the date. Label the third tube "control."
2. **Inoculate the Media:** Use an inoculating needle to stab inoculate the appropriate sample tubes. For best results, use a heavy inoculum, an amount of bacteria roughly equivalent to a couple of colonies. Do not inoculate the control.
3. **Incubate Your Samples:** Incubate all tubes at 25°C for up to 1 week. Your instructor may direct you to incubate the samples at a higher incubation temperature (e.g., 35–37°C), in which case you will need to refrigerate the samples and the uninoculated control before making your observations.
4. **Clean up:** Dispose of stock samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

→ Next Lab Session

1. **Obtain Your Samples:** Put on your lab safety gear, and then collect your cultures from the incubator (or wherever your instructor placed your samples). If the uninoculated control is liquified, then all liquified samples must be refrigerated or placed in an ice bath before making observations. If the uninoculated control is solid, the test results for the other samples can be read without refrigeration.
2. **Examine Your Samples:** When the control has solidified (it shouldn't take more than 15–20 minutes, but it must solidify before proceeding), examine the inoculated media for gelatin liquefaction. Carefully tilt the tubes to determine if the media is liquid or solid. Be careful that you do not spill liquified gelatin when tipping the tubes. If you

do spill the media, follow your lab's clean up protocols and disinfect the space before proceeding.

3 **Record Your Result:** Refer to Figure 39.2 and Table 39.1 when interpreting your results. Record your results in the table provided in the data sheet at the end of this exercise.

4 **Clean up:** Dispose of the samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 39

DATA SHEET

Gelatin Hydrolysis (Gelatinase Test)

During the Lab

1 Using Table 39.1 and Figure 39.2 for guidance, enter your findings in the following table.

Organism	Observations	Test Result (+/–)	Interpretation
Control			

Lab Related Questions

1 Choose the true statement(s) about the enzyme(s) detected in this test (select all that apply).

a. They are exoenzymes.

b. They are hydrolytic.

c. They are inactive at body temperature.

d. They break down amino acids.

e. They are proteins.

2 You stab inoculate 10 solidified tubes of nutrient gelatin, each with different bacteria, and leave one of the tubes uninoculated. You then incubate each sample at 25°C for a week. Upon making your observations, you notice that all the tubes are liquified, including the uninoculated control. Which of the following is the most likely explanation for the results?

a. The media was prepared with too little gelatin.

b. The media was contaminated during preparation.

c. You used too much inoculum when you inoculated the samples.

d. Each of the tested bacteria make gelatinase.

e. You accidentally inoculated what was supposed to be the uninoculated control.

3 Select the scenarios that could lead to a false-negative test result (select all that apply).

a. You placed the sample in the fridge for a full day.

b. You inoculated a sample twice with the same bacterium.

c. You didn't incubate the samples long enough.

d. You forgot to chill the sample before analyzing the test result.

e. You shook the warm tubes before placing them in the refrigerator.

4 Choose the true statement(s) about the gelatinase test (select all that apply).

a. The media used in the test is selective.
b. It is a differential test.
c. It can help distinguish *Staphylococcus aureus* from *S. epidermidis*.
d. For a positive test result, the media must be fully liquified.
e. The media liquifies at room temperature.

5 Assume you inoculate four tubes of nutrient gelatin with a pure culture of the same bacteria. After 2 days you observe that half of the inoculated samples are partially liquified, but half of your samples remain completely solid. The uninoculated control remained solid. What is the best explanation for your observations?

a. In half of the samples, the tested bacteria mutated and lost the ability to make gelatinase(s).
b. The liquification was due to increased temperature rather than gelatinase activity.
c. The tested bacterium is gelatinase negative.
d. The inoculated tubes that remained solidified were underinoculated.
e. The gelatin media was improperly prepared.

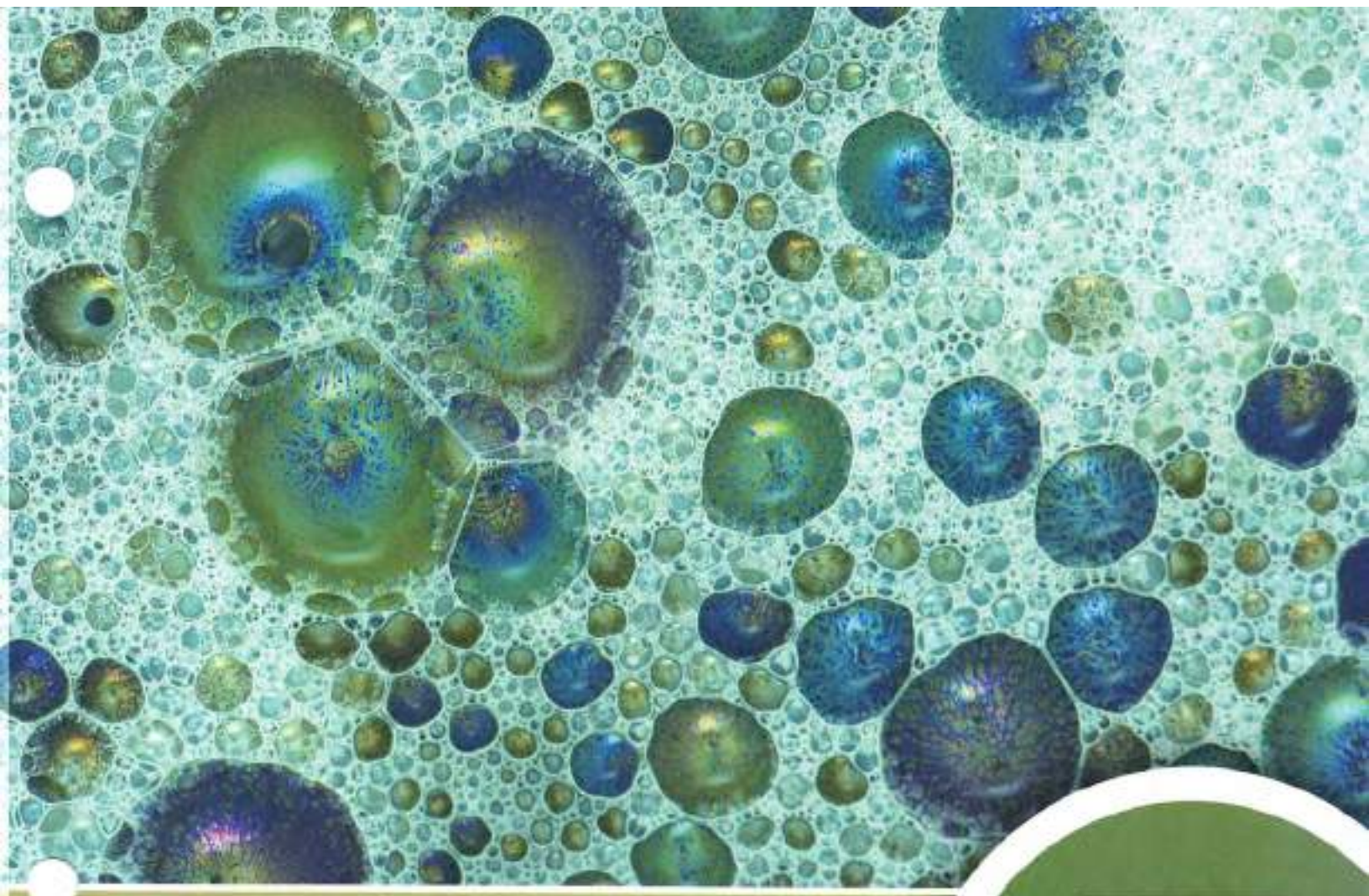
6 Choose the true statement(s) (select all that apply).

a. Gelatinases may allow bacteria to invade host tissues to establish an infection.
b. Gelatinases may allow bacteria to live at warmer temperatures.
c. All pathogens make gelatinases.
d. *Enterobacteriaceae* are usually gelatinase negative.
e. Gelatinases are usually most active at the bottom of the inoculated tube.

Critical Thinking

1 If the control is solid and an inoculated tube is liquid, is it acceptable to read the result before the complete incubation time has elapsed? Explain your reasoning.

2 What effect would doubling the amount of gelatin in the media have on test sensitivity?



SECTION

7

Controlling Bacterial Growth

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— Lab Notes —

EXERCISE 40

A Comparison of Hand-Cleansing Agents[1]

After doing this exercise, you should be able to:

1. Define sanitization and state how it differs from sterilization.
2. Discuss the differences between disinfectants and antiseptics.
3. Explain the proper method of hand washing for general patient contact.
4. State the goal of antisepsis washing.
5. Describe the difference between microbiocidal and microbiostatic agents.
6. Describe the three tiers of germicides.
7. Outline and evaluate an experimental design intended to illustrate the relative effectiveness of assorted hand-cleansing agents.

Background

Microbiological laboratory techniques and safe health care both depend on sanitization and sterilization procedures. In prior labs, we defined **sterilization** as a process that eliminates all microbial agents from a substance. In contrast, here we discuss the practice of **sanitization**, a process that reduces the number of microbes on a living or nonliving surface. In general, most germicides achieve sanitization; only high-level germicides, which we'll discuss later in this lab, can achieve sterilization.

There are two main categories of germicides: **disinfectants**, which we can use on nonliving surfaces (countertops, equipment, etc.), and **antiseptics**, which we can apply to living tissue such as skin. In this exercise you will compare the relative effectiveness of commercially available *antiseptics* used to sanitize hands (or skin in general) to reduce bacteria levels.

The concept of proper hand hygiene as protective against infections has gone from a controversial beginning (in the early 1800s) to an accepted practice. However, only properly performed hand washing is effective at preventing disease transmission, thus it's important to understand exactly what proper hand washing entails.

What is Proper Hand Washing?

The COVID-19 pandemic brought hand washing into the public health spotlight and led many to wonder how and when to wash up. The U.S. Centers for Disease Control and Prevention (CDC) advised that hand washing should be performed "after being in public and touching an item or surface that may be frequently touched by other people" as well as "before touching your eyes, nose, or mouth."

The hand-washing approach used depends on the goal. For example, hand washing before surgery (surgical scrubbing) to achieve **antisepsis** (a reduction of the normal resident microbiota, the elimination of transient microbes picked up by normal daily functioning, and the blocking of rapid rebound growth of microbes) is far more rigorous than the basic hand washing pursued before and after a general patient encounter. The steps for general patient encounter hand washing are as follow:

1. Wet your hands with cold or warm water.
2. Then, into the palm of your hand, apply the amount of cleansing product the manufacturer recommends.
3. Vigorously rub your hands together for at least 20 seconds. Be sure to cover all surfaces of your hands and wash well between your fingers and up your wrists.
4. Use a disposable towel to dry your hands.
5. Use the same towel that you used for drying to turn off the water.

What Works Best?

In the early stages of the COVID-19 pandemic, there was a shortage of alcohol-based hand rubs to limit transmission of the SARS-CoV-2 virus. This prompted health officials to remind people that proper hand washing with soap and water is just as effective at reducing disease transmission as alcohol-based agents. But the question, "What works best?" remains an interesting topic to explore since so many hand-cleansing agents are commercially available.

In this exercise, you will compare the relative effectiveness of several hand-cleansing agents, each of which has its own appropriate application. Some are for routine household use, such as "washing up before dinner." Others, while available to the public, are best reserved for clinical settings where infection prevention is a matter of life and death. And yet others fall somewhere in between. Figure 40.1 shows examples of products that we test in this exercise.

[1] Thanks to reviewers Johana Meléndez-Santiago and Janice Yoder-Smith for their suggestions on developing this laboratory exercise.

No matter what the product is, each contains a germicide that is either **microbiocidal** (kills certain microbes) or **microbiostatic** (inhibits the growth of certain microbes).[2] A germicide's decontamination efficacy is ultimately due to its ability to damage and/or inactivate essential cellular components. The more damage the germicide does to the microbial target, the higher the decontamination potential. The cell component targeted could be as small as an enzyme, as large as the entire cytoplasmic membrane, or anything in between.

In general, germicides fall into three categories. High-level germicides destroy all bacteria, fungi, viruses, and low levels of bacterial endospores thereby rendering objects sterile. Intermediate-level agents have the same span of action as high-level agents, but are not effective against bacterial endospores and therefore are not sterilizing agents. Similarly, low-level germicides are not sterilizing compounds; despite destroying most bacteria, fungi, and viruses, they are not effective against bacterial endospores or *Mycobacterium* species.

[2] The suffix "cidal" means killing where as "static" means inhibitory. Thus, an agent that is fungicidal and bacteriostatic will kill fungi, but only inhibit bacterial growth rather than kill bacteria.

Clinical Applications

It is a well-established fact that hand washing reduces disease transmission and good hand hygiene can help *everyone* stay healthier by reducing exposure to bacterial and viral pathogens. However, when it comes to health-care workers, food handlers, and laboratory personnel, proper hand-washing techniques can save lives. Thus, training in how to properly perform hand washing is important.

When health-care workers follow proper hand-washing methods they protect themselves from pathogens found in health-care settings and they protect patients from **health care-associated infections (HAIs)**, infections patients may develop while receiving treatment for an unrelated medical condition. Similarly, food handlers can limit foodborne illness transmission by frequent and proper hand washing. Lastly, by employing proper hand-washing methods, laboratory workers who handle pathogens (or potential pathogens) can minimize self-inoculation, reduce the risk of spreading infectious agents to others, and lower the chances of culture contamination.

FIGURE 40.1 Hand-Cleansing Agents • From left to right are: Betadine scrub (10% povidone iodine), alcohol-based hand sanitizer (65% ethyl alcohol), chloride-based hand sanitizer (0.13% benzalkonium chloride), antibacterial soap (0.13% benzalkonium chloride), and Hibiclens (4% chlorhexidine gluconate). This exercise uses these agents, but others can be substituted, such as Dawn antibacterial dish soap (0.3% chloroxyenol). If a student has skin sensitivities, they can perform the exercise with just tap water.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Nutrient agar (NA) or tryptic soy agar (TSA) plate (1)
- ❑ Access to a sink and paper towels for hand washing
- ❑ Permanent marking pen
- ❑ Bottles of as many as 5 of the following or suitable replacements*:
  - ☐ Antibacterial liquid soap containing 0.3% chloroxyenol (e.g., Dawn antibacterial dish soap)
  - ☐ Alcohol-based hand sanitizer containing at least 60% ethyl alcohol (ethanol)
  - ☐ An alcohol-free hand sanitizer containing 0.13% benzalkonium chloride (e.g., Hy5 brand of hand sanitizer)
  - ☐ Betadine scrub (10% povidone iodine)
  - ☐ Hibiclens (4% w/v chlorhexidine gluconate)

* Students should not ingest or get any of these products in their eyes. Follow package inserts for agent use. Students should not work with agents for which they have a known allergy or sensitivity.

PROCEDURE

In this exercise, you will evaluate the relative effectiveness of a variety of hand-washing antiseptics. Although you will not wear gloves while sampling your fingers during the first lab session (the session where this lab is set up), you should wear your other safety gear: your lab coat and eye protection. During the second lab session, when you make your growth observations, wear your lab coat, eye protection, *and* gloves. It's important to note that you may have grown a pathogen in this experiment. As such, follow all BSL-2 precautions when handling the plates in your second lab session. To reduce exposure to possible BSL-2 level bacteria, keep all plates *closed* during the second laboratory session.

CAUTION!

The U.S. Centers for Disease Control and Prevention cautions, "Either nonpathogenic or attenuated bacterial strains should be used when possible, especially in teaching laboratories." Before assigning this exercise, faculty should check institutional policies regarding cultivating unknown bacteria from environmental sources and from human subjects. Anytime such sampling is performed, BSL-2 agents that are *not* attenuated could be isolated. Faculty are strongly cautioned to review their institution's policies on students handling such samples. This lab exercise should only be performed in laboratories approved to handle BSL-2 agents.

1. **Obtain and Label the Media:** Label the base of a nutrient agar or tryptic soy agar plate with your name and the date. Then use the marking pen to draw a line down the middle of the plate, thereby dividing the plate in half. Label one half "Before" and the other half "After." Then, write the numbers 1 through 5 on each side of the dividing line so the numbers line up. That is, number 1 on the "Before" side should be opposite number 1 on the "After" side, and so on. Spread the numbers out from one another as much as possible since you will eventually press a finger to each number.
2. **Get Your Hands Dirty:** Sample an area of your choosing by rubbing your left-hand fingertips (all five of them) against the target area. Ideas for sampling include the floor, a tabletop, your backpack, or your forehead.
3. **Inoculate the Medium:** Then, one at a time, gently press your thumb tip next to the "Before" number 1, your index finger next to the "Before" number 2, and so on. Your middle, ring, and pinky fingers are numbered 3, 4, and 5, respectively.
4. **Wash Your Hands:** Wash your hands with soap and water.
5. **Record the Names of the Agent(s) You'll Test for Each Finger:** Use the data sheet at the end of this exercise to record the hand-cleansing agent(s) you will apply to each finger. You will assign a cleansing agent to each finger of your right hand (e.g., thumb-1= triclosan; index finger-2 = alcohol-based hand sanitizer, etc.)
6. **Testing the Cleaning Agent(s):** Now sample the same site used in Step 2 with the fingertips of your *right* hand, but this time you'll only "dirty" one fingertip at a time. Then, you will rub the dirtied fingertip in a few milliliters of the hand-cleansing agent in your left palm (see Steps a and b for further directions). If the cleansing agent requires rinsing and drying, perform this at a sink. Agents that only require air-drying can be applied/tested at your table.
   a. Place a quarter-size drop of the first hand-cleansing agent in the palm of your left hand. Next, dirty your right thumb by rubbing it on the area to be sampled. Then, rub your dirtied thumb into the cleansing agent for 10 seconds.[3] Once your thumb is dry, gently press it next to the number 1 on the "After" side of your plate. Wash both hands with soap and water, and dry them.

[3] The recommended time for hand washing is about 20 seconds, but because you are only washing the fingertips and concentrating on those, that time has been reduced to see representative results.

b To test the remaining four hand-cleansing agents, repeat Step a using a different finger for each cleansing agent. Be sure to press each finger onto the agar next to its correct number and to wash your hands between each surface sampling step.

7 **Seal and Incubate Your Plate:** Seal your test plate by taping the lid to the base. Then, incubate your plate at 25°C (slightly warmer than average room temperature) until your next lab session.

8 **Clean up:** Dispose of the materials as your instructor directs, disinfect your work surface, and thoroughly wash your hands with soap and water before leaving the lab.

→ Next Lab Session

1 **Examine the Samples:** Examine your plates for growth. (*Note:* Do not remove the plate's lid unless given permission to do so.) Each colony you see is likely the product of a single cell that was deposited onto the agar. Different microbial species often produce distinctly different colonies, so note colony diversity by inspecting colony features such as color, visual sheen, etc. Refer to Exercise 6 for a review of colony morphology features.

2 **Record Your Observations:** Fill in the table provided at the end of this lab exercise as you assess the relative amount of growth and microbial diversity (based on colony differences) on the "Before" and "After" sides of the plate for each agent. Recognize that you are comparing "apples and oranges" with respect to the intended uses of each cleansing agent. That is, the products tested range from surgical scrubs to over-the-counter soaps intended for household use. A surgical scrub may have better antimicrobial activity than hand soap, but that doesn't make it "better" for household use!

3 **Clean up:** Dispose of the plates as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 40

DATA SHEET

A Comparison of Hand-Cleansing Agents

During the Lab

1 To complete the **relative growth** column in the table provided, use this qualitative scale for evaluation:

+++ means "a lot of growth"

++ means "moderate growth"

\+ means "little growth"

0 means "no growth"

Note: There is not an absolute cutoff between any of these categories. What you call "moderate growth" might be called "little growth" by another student. Just try to be consistent within your evaluation.

For recording colony **diversity**, look for different colony colors, shapes, textures, sizes, and anything else that is indicative of a different organism growing, and record the number of different organisms present. For the **interpretation** column, describe the relative effectiveness of the tested agent (excellent, good, fair, poor).

Number on Plate	Hand-Cleansing Agent	"Before"		"After"		Interpretation
		Relative Growth	Diversity	Relative Growth	Diversity	
1						
2						
3						
4						
5						

Lab Related Questions

1 Which of the following represents a breach in proper hand-washing protocols for general patient encounters? (Select all that apply.)

a. Failing to wash after the patient encounter.

b. Washing for 10 seconds.

c. Turning on the faucet with a bare hand before hand washing.

d. Turning off the faucet with a bare hand after hand washing.

e. Washing between the fingers and up the wrists.

2 If you were to reuse the same finger for each cleansing agent, which of the following would you most likely observe? (Select all that apply.)

a. The bacterial growth observed for the last agent would be the lowest regardless of the nature of the cleansing agent.
b. The bacterial growth observed for the last agent would be the highest regardless of the nature of the cleansing agent.
c. The bacterial diversity observed for the last agent would be the highest regardless of the nature of the cleansing agent.
d. The bacterial diversity observed for the last agent would be the lowest regardless of the nature of the cleansing agent.
e. You would not observe bacterial growth for any of the cleansing agents.

3 Define the term *microbiostatic*.

4 Define the term *microbiocidal*.

5 Select the true statement(s) (select all that apply).

a. The less damage that a germicide does to a cell, the higher the decontamination potential.
b. Hand washing reduces HAI transmission.
c. A bactericidal agent will lead to less bacterial growth on the experimental plates in the exercise as compared to a bacteriostatic agent.
d. A bactericidal agent is less likely to kill bacteria than a bacteriostatic agent.
e. If hand washing with soap and water is properly performed, it is just as effective at reducing disease transmission as alcohol-based hand sanitizers.

6 Select the true statement(s) (select all that apply).

a. Sterilization is the removal of all microbial agents from a substance.
b. Antisepsis washing achieves sterilization.
c. Sanitization is the same as sterilization.
d. High-level germicides can sterilize a surface.
e. Hand washing does not achieve sterilization.

7 Which of the following would be appropriate for use on a living surface? (Select all that apply.)

a. Germicides
b. Disinfectants
c. Antiseptics
d. Microbiocidal agents
e. Microbiostatic agents

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 40 DATA SHEET

(continued)

8 Select the false statement about antisepsis washing.

a. It leads to a permanent reduction in normal microbiota levels.

b. It removes transient microbiota from the hands.

c. It is a form of hand sanitization.

d. It is performed before surgery.

e. It blocks the rapid rebound growth of microbes on the area treated.

9 Which of the following would you expect for a low-level germicide? (Select all that apply.)

a. It would destroy bacterial endospores.

b. It would not achieve sterilization.

c. It would not eliminate *Mycobacterium tuberculosis* from a surface.

d. It would eliminate most viruses.

e. It would achieve sanitization.

Critical Thinking

1 What other variables besides the cleansing agent might be responsible for any differences noted between fingers or students?

2 Do some research and fill in the following table. Your answer to "Antibacterial Effect" should address the structural and/or functional part of the cell affected, as well as whether the agent is bactericidal (kills bacteria) or bacteriostatic (stops bacterial growth, but doesn't kill bacteria). Under "Other Antimicrobial Effects," note any antiviral, antiprotozoal, and/or antifungal properties of the agent.

Active Ingredient	Antibacterial Effect	Other Antimicrobial Effects
Chloroxyenol		
Isopropanol/ethanol (isopropyl/ethyl alcohol)		
Benzalkonium chloride		
Povidone iodine scrub		
Chlorhexidine gluconate		

EXERCISE 41

Effectiveness of Chemical Germicides: The Use-Dilution Test for Disinfectants and Antiseptics[1]

After doing this exercise, you should be able to:

1. Define the term *germicide*.
2. Define the term *disinfectant*.
3. Define the term *antiseptic*.
4. Describe the application and theory behind this procedure.
5. Perform and interpret the results of this procedure.

Background

As reviewed in Exercise 40, **germicides** are substances that reduce microbial levels on surfaces. They include **disinfectants** for use on nonliving surfaces (e.g., countertops) and **antiseptics** for use on living surfaces (e.g., skin). Also, we reviewed that based on their degree of efficacy, there are three general categories of germicides: high-level, intermediate-level, and low-level agents. Of the three germicide tiers, high-level germicides are the most effective against bacterial endospores, dormant structures that certain spore-forming bacteria such as *Bacillus*, *Clostridioides*, and *Clostridium* genera make. Before a new substance is marketed, the Food and Drug Administration (FDA) and the environmental protection agency (EPA) require that the agent is tested and classified according to its effectiveness against pathogens. The Use-Dilution Test, published by the Association of Official Analytical Chemists (AOAC), is one of many commonly used tests for this purpose.

The Use-Dilution Test is a standard procedure used to measure the effectiveness of disinfectants specifically against *Staphylococcus aureus*, *Salmonella enterica* serovar Choleraesuis, and *Pseudomonas aeruginosa*. In the standard procedure, glass beads or stainless-steel cylinders coated with living bacteria are exposed to varying concentrations (dilutions) of test germicides and then transferred to a growth medium.

After an incubation period, the medium is examined for growth. If a solution prevents microbial growth at least 95% of the time, then it meets the required standards and is considered a usable dilution of that germicide for a specific application. Today's exercise is an adaptation of this method.

The Experimental Process

Today you will examine the effectiveness of four germicides: two common household disinfectants (bleach and Lysol Brand II Disinfectant) and two over-the-counter antiseptics (hydrogen peroxide and isopropyl alcohol). The standard test bacteria are BSL-2 agents, but here you'll perform the protocol with BSL-1 bacteria: *Staphylococcus epidermidis* and *Escherichia coli*.

First, you will coat presterilized ceramic or glass beads with bacteria. You will then expose the coated beads to three concentrations of your assigned germicide. After germicide exposure, you will use the treated beads to inoculate sterile nutrient broth samples. If all the bacteria on the bead are killed during exposure to the germicide, the broth inoculated with that bead will remain clear. If any of the bacteria survive the germicide exposure, they will reproduce during incubation and turn the broth turbid (cloudy). Based on your observations, you will be able to determine the effective concentration (dilution) of your assigned germicide.

The tasks for the exercise are divided among eight student groups (or a convenient number for your lab size). Each group will be responsible for one organism and three dilutions of a specific germicide. Refer to Table 41.1 for your group assignment.

TABLE 41.1 Group Assignments •

Germicide	*Staphylococcus epidermidis*	*Escherichia coli*
Bleach	Group 1	Group 2
Lysol	Group 3	Group 4
Hydrogen peroxide	Group 5	Group 6
Isopropyl alcohol	Group 7	Group 8

Finally, this is an interesting exercise with a moderate amount of work involved. If you take your time and carefully apply your aseptic techniques, you will be rewarded with reliable data.

[1] This procedure is modified from its original form and should therefore only be used for instructional purposes.

Clinical Applications

The procedure presented is a modification of the standard Use-Dilution Test protocol used to test the effectiveness of germicides against *Staphylococcus aureus*, *Salmonella enterica* serovar Choleraesuis, and *Pseudomonas aeruginosa*. These are all pathogens that can be found in health-care settings. Knowing what germicides work best against a specific pathogen and knowing the necessary concentrations for maximal efficacy is important to promote effective sanitization procedures. Guarding patient health relies heavily on proper sanitizing of medical equipment and clinical spaces.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Timer or stopwatch
- ❑ 100 mL flask of sterile deionized water
- ❑ Concentrations (3) of one germicide (listed in Table 41.1)
- ❑ Sterile 60 mm petri plates (5)
- ❑ Sterile glass 100 mm petri plate containing filter or bibulous paper (1)
- ❑ Container of sterile ceramic or glass beads* (1)
- ❑ Sterile transfer pipette
- ❑ Sterile nutrient broth (NB) tubes (7)
- ❑ Needle-nose forceps (or appropriate device for aseptically picking up beads)
- ❑ Small screw-cap jar with alcohol (for flaming forceps)
- ❑ Small beaker containing 10 mL of disinfectant (for broth culture disposal)
- ❑ Fresh broth cultures of these recommended organisms (only 1 per group):
  - ☐ *Escherichia coli*
  - ☐ *Staphylococcus epidermidis*

  Disinfectants
  - ☐ 0.01%, 0.1%, and 1% household bleach
  - ☐ 25%, 50%, and 100% Lysol Brand II Disinfectant

  Antiseptics
  - ☐ 0.03%, 0.3%, and 3% hydrogen peroxide (3% is full strength as purchased at the pharmacy)
  - ☐ 10%, 30%, and 50% isopropyl alcohol (70% or 90% is full strength as purchased at the pharmacy)

* Sterilized #8 seed beads from a craft store will work for this purpose.

PROCEDURE

Timing is important in this procedure. Read through the entire procedure before you start. You need to develop a plan with your group *before* you begin your transfers. Having a plan will make it more likely that you'll have uniform soaking times and processes consistent with what other groups are doing. During the lab exercise, you should wear your lab coat, eye protection, and gloves.

1. **Obtain and Label the Media:** As shown in the diagram in Figure 41.1 (Step 1) obtain seven tubes of broth media and label each with your group name/number. Label three of the broths "Broth #1, #2, and #3" and four broths "Control #1, #2, #3, and #4."
2. **Obtain Your Stock Culture:** Your group was assigned a specific bacterium to test, either *Staphylococcus epidermidis* or *Escherichia coli*. Obtain the stock culture and note the name of the organism here: _____.
3. **Set up Your Treatment Stations:** Your group was assigned a specific disinfectant or antiseptic to test. Write down the name of the agent your group will test: _____. Now, obtain five sterile 60 mm petri plates. Label three of the plates with the three test dilutions of your germicide. Label the remaining two plates "Sterile Water." Add enough of each germicide concentration to its respective plate (Plates #1 through #3) to cover the beads (approximately 15 mL). Add an equal volume of sterile water into Plates #4 and #5.
4. **Set up Controls 1 and 2:** Per Figure 41.1 (Step 2), carefully mix your bacterial culture until uniform turbidity is achieved. Take care not to splash into the cap. Use an inoculating loop to transfer a loopful of bacteria from the stock sample into Control Tube #2. Control Tube #1 is your uninoculated control, so just set it aside along with the inoculated Control Tube #2.
5. **Coat the Beads with Bacteria:** Per Figure 41.1 (Step 3), alcohol-flame your forceps and aseptically drop six beads into the stock culture. (You will only use four beads; the other two are extras in case you drop one during the procedure.) After the beads have soaked for 1 minute, decant the liquid portion into a beaker of disinfectant. Remove as much of the broth as possible without losing the beads in the disinfectant. Then, so that the beads can dry, carefully pour the beads onto a piece of filter paper in a sterile 100 mm petri plate. Allow the beads to air-dry for 10 minutes.

CAUTION!

Store your forceps in the alcohol jar between transfers. When it is time to make a transfer, remove them from the alcohol. Then pass them through the flame to burn off the alcohol, holding them away from the alcohol jar while doing so. If the alcohol jar catches on fire, smother the flame with the lid. When finished, return the forceps directly to the jar without flaming.

6 **Treat the Beads:** Transfer one bacteria-coated bead from Step 5 to each treatment as shown in Figure 41.1 (Step 4A). Then, as shown in Figure 41.1 (Step 4B), re-flame the forceps and transfer a sterile bead (not a bacteria-coated bead) to a separate plate of sterile water that has not been used to treat bacteria-coated beads (Plate #5 in your set up). Allow the beads to soak in their respective treatments for 10 minutes.

7 **Add the Treated Beads to Your Broth Tubes for Incubation:** As shown in Figure 41.1 (Step 5), use flame-sterilized forceps to transfer each bead to a separate tube as indicated.

8 **Incubate the Tubes:** Once all the beads have been transferred to their respective broth tubes, incubate all seven tubes at 35±2°C for 48 hours.

9 **Clean up:** Dispose of the materials as your instructor directs, disinfect your work surface, and thoroughly wash your hands with soap and water before leaving the lab.

→ Next Lab Session

1 **Examine the Samples:** Remove all broth tubes from the incubator. Gently mix Control #1 and Control #2 and examine them for evidence of growth. Enter your results on the data sheet at the end of this exercise. (*Note:* Control #1 should have no growth and Control #2 should show turbidity. If both conditions are met, you may proceed with your comparison. If not, see your instructor.)

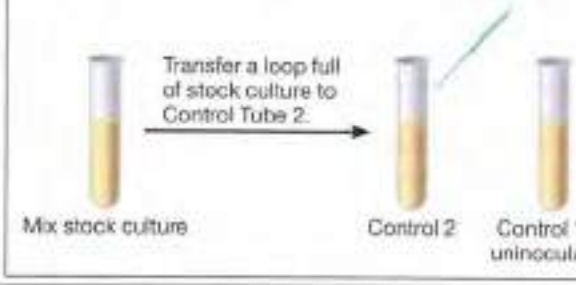
2 **Record Your Observations:** Using Controls #1 and #2 as comparisons, examine broths containing beads exposed to germicide (Broths #1, #2, and #3) and examine Controls #3 and #4. Using "G" to indicate growth and "NG" to indicate no growth, enter your results in both the individual data table and the class data table on the data sheet at the end of this exercise.

3 **Record Data from Other Groups:** Your instructor will provide you with a means to share data obtained by all eight groups. Record these data on your data sheet at the end of this exercise.

4 **Clean up:** Dispose of the samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

1. Label 7 tubes with your group name and the labels as shown.

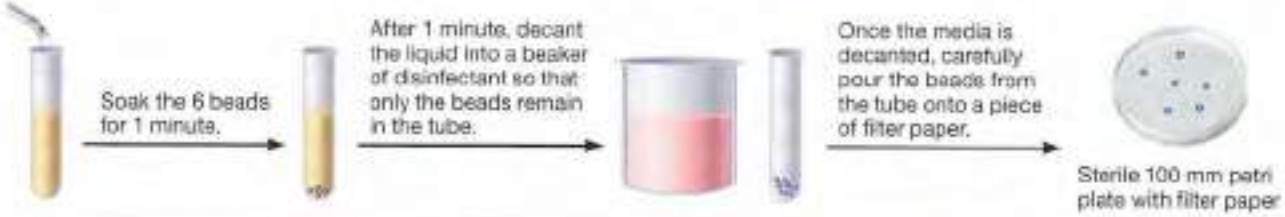
2. Mix your stock culture and inoculate Control 2. Also, set Control 1 aside as your uninoculated control.



Set Controls 1 and 2 aside until it is time to incubate all the tubes.

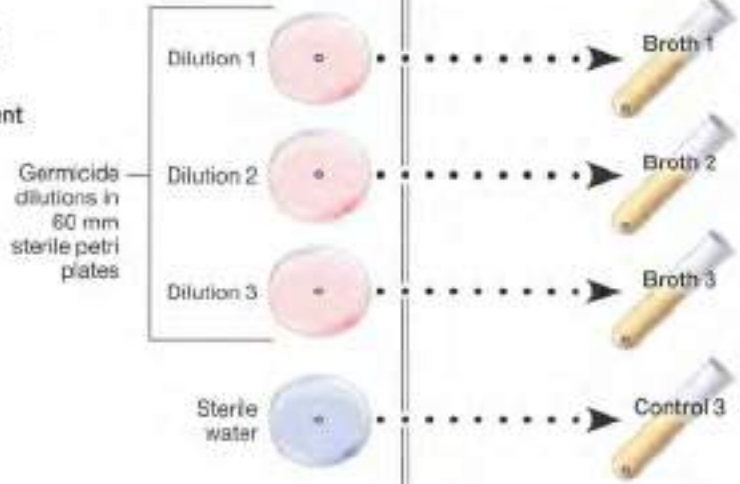
3. Coat the pre-sterilized beads with bacteria and then transfer them to filter paper to dry.

Use flame-sterilized forceps to add 6 sterile beads to the stock culture. You'll only use 4 beads, but the others are in case you drop a bead.


Soak the 6 beads for 1 minute.
After 1 minute, decant the liquid into a beaker of disinfectant so that only the beads remain in the tube.
Once the media is decanted, carefully pour the beads from the tube onto a piece of filter paper.
Sterile 100 mm petri plate with filter paper

Once the 6 beads are on the paper, let them air-dry for 10 minutes. Four of these beads will be transferred to treatments (germicide dilutions and sterile water) in the next step.

4A. Flame-sterilize the forceps and transfer one bacteria-coated bead from the prior step to each treatment as shown.



4B. Re-flame the forceps to sterilize them. Then transfer a *sterile bead* (not a bacteria-coated bead) to a separate plate of sterile water that has not been used to treat bacteria-coated beads.

Once all beads are in their designated treatment, set the timer for 10 minutes.

5. After treating the beads in germicide or water for 10 minutes, use flame-sterilized forceps to transfer each to a separate tube as indicated.

NOTE: **Flame-sterilize the forceps between every bead transfer.**

Incubate all tubes (including Controls 1 and 2 from Step 2) at 35°C for 48 hours.

FIGURE **41.1** **Procedural Diagram for Chemical Germicides**

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 41

DATA SHEET

Effectiveness of Chemical Germicides: The Use-Dilution Test for Disinfectants and Antiseptics

During the Lab

1 Enter your individual data in the following table.

Controls	Growth (G) or No Growth (NG)
#1	
#2	
#3	
#4	

2 Enter your data as well as other group data in the following table. Use "G" for "Growth" and "NG" for "No Growth."

Organism	Household Bleach			Lysol Brand II Disinfectant			Hydrogen Peroxide			Isopropyl Alcohol		
	0.01% Broth 1	0.1% Broth 2	1% Broth 3	25% Broth 1	50% Broth 2	100% Broth 3	0.03% Broth 1	0.3% Broth 2	3% Broth 3	10% Broth 1	30% Broth 2	50% Broth 3
S. epidermidis												
E. coli												

Lab Related Questions

1 Select the true statement(s) about antiseptics (select all that apply).

a. Disinfectants can be used as antiseptics.

b. Antiseptics are germicides.

c. Antiseptics may be used on skin.

d. The same antiseptic may be effective against one microbial species at one concentration but require a different concentration to be effective against a different species.

e. Bleach is considered an antiseptic.

2 Which of the following should lack growth? (Select all that apply.)

a. Control #1
b. Control #2
c. Control #3
d. Control #4

3 A student failed to allow the coated beads to dry before transferring them to the germicide treatment. Which of the following experimental outcomes would be *most* likely to occur due to this error?

a. Moisture from the beads would overly dilute the germicides and render the treatments ineffective for all the tested samples.
b. None of the germicide concentrations would appear to be effective because the bacterial levels on the beads would be too high.
c. The moisture would prevent the germicide from interacting with the bacteria on the bead thereby leading us to conclude the germicide was less effective than it truly is.
d. The bacteria would wash off the beads as soon as they were placed in the germicide leading to less growth even if the germicide was not effective against the test bacteria.
e. An effect of this error is unlikely to occur.

4 Select the false statement about the Use-Dilution Test.

a. All the beads are sterile at the start of the test.
b. The test bacteria used in the exercise are the same as used in the standard protocol.
c. The test is used to measure the effectiveness of disinfectants.
d. The test requires use of a pure bacterial culture.
e. The test helps manufacturers comply with FDA and EPA standards.

5 Which of the following scenarios would you be *most* likely to encounter if you forgot to flame-sterilize the forceps between transferring each bead to the tubes of broth?

a. Broth Tube #2 may exhibit growth even if Broth Tubes #1 and #3 did not.
b. Broth Tube #2 may exhibit growth even if Broth Tube #3 did not.
c. Broth Tube #3 may exhibit growth even if Broth Tube #1 did not.
d. Only Control Tube #2 would exhibit growth.
e. All the control tubes would exhibit growth.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 41
DATA SHEET
(continued)

Critical Thinking

1 Compare your results with the class data.

a. Which germicide was most effective and at what concentration? Defend your choice.

b. Which was least effective? Defend your choice.

2 Which organism seemed to be more resistant to the germicides?

EXERCISE 42

The Effect of Ultraviolet Radiation on Microbial Growth

After doing this exercise, you should be able to:

1. Describe some basic principles regarding UV light and state why UV-C is germicidal.
2. State some pros and cons of UV decontamination methods.
3. Discuss the application and theory behind this procedure.
4. Perform and interpret the results of this procedure.

Background

In Exercises 40 and 41, we reviewed chemical germicides, disinfectants, and antiseptics as agents that help us reduce microbial levels. In this exercise, you will examine control of microbial growth by physical means. Examples of physical microbial control include temperature changes (heat/cold), radiation, and filtration. Here we will illustrate the germicidal effects of ultraviolet (UV) radiation.

UV radiation is a type of **electromagnetic energy**. Like all electromagnetic energy, UV travels in waves and is distinguishable from all others by its **wavelength**. Wavelength is the distance between adjacent wave crests and is typically measured in nanometers (nm) (Fig. 42.1). Based on wavelength, ultraviolet light is divided into three groups:

1. UV-A, the longest wavelengths, ranging from 315 nm to 400 nm;
2. UV-B, wavelengths between 280 nm and 315 nm; and
3. UV-C, wavelengths ranging from 100 nm to 280 nm.

Of these three categories, UV-C is the most detrimental to microbes. Bacterial exposure to UV-C (specifically 240–280 nm) for more than a few minutes usually results in irreparable DNA damage and cell death. A few minutes of UV-C exposure also tends to irreversibly damage viral genetic material, making these disinfection methods broadly useful to protect against viral contamination. For example, during the earlier stages of the COVID-19 pandemic, N95 respirator masks were in short supply, so many health-care workers periodically placed their masks under UV light so they could get multiple uses out of what would typically have been a single-use mask. The germicidal effect of UV-C is related to time of exposure, lamp intensity, and distance to the target (intensity *decreases* as distance from the source to the target *increases*). The UV

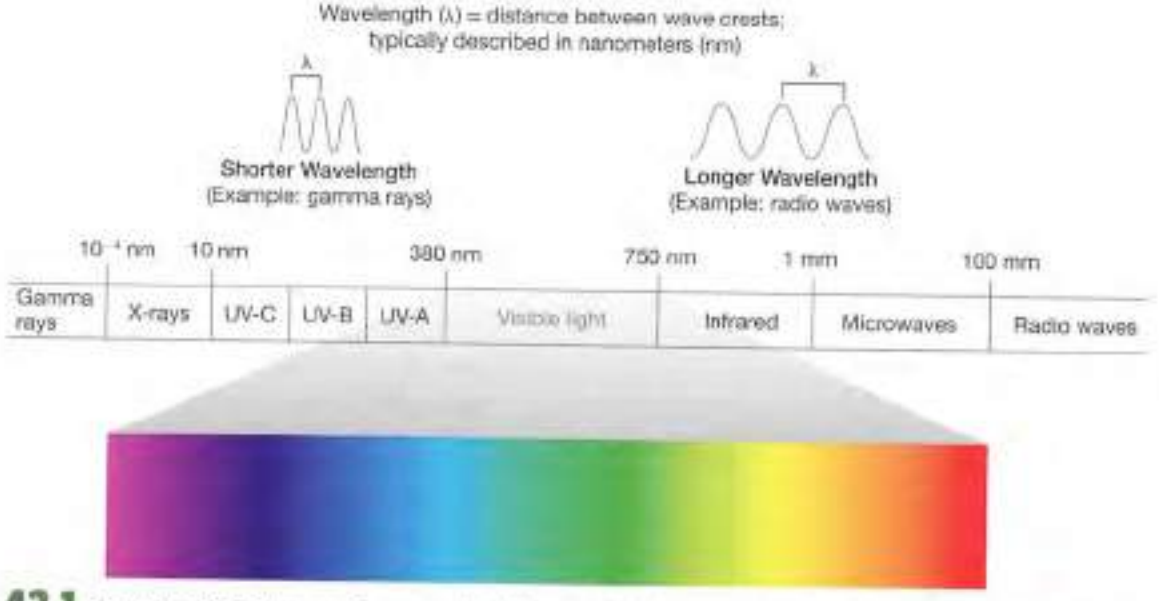


FIGURE **42.1 Electromagnetic Spectrum** • Gamma rays have the shortest and highest energy wavelengths (λ less than 10^{-4} nm). In contrast, radio waves have long wavelengths that can be 1 kilometer or longer. Between about 100 nm and 380 nm (just shorter than visible light) is the portion known as ultraviolet light.

light must also have "line of sight" to the surface being decontaminated. That is, UV-C doesn't penetrate materials well nor does it readily bend around corners or trickle into crevices. This means substances such as dust, organic matter, or any other material that may shield microbes from direct exposure to the UV-C waves will diminish the disinfecting efficacy of a UV treatment.

Lastly, the exposure time and intensity of the UV treatment must be optimized for the microbes being targeted. For example, a UV treatment that may eliminate certain viruses may not destroy bacterial endospores, hearty structures that are challenging (though not impossible) to destroy via chemical and physical methods. Taken together, this means that UV-C as a germicidal agent has its limited applications. However, the upsides of UV-C as a decontamination measure are also worth noting. Namely, UV-C decontamination approaches are inexpensive, these measures do not require the storage of chemicals that can expire or spill, and UV-C can decontaminate air and water without introducing chemicals into the environment.

The Experimental Process

The tasks for the exercise are divided among six student groups (or a convenient number for your lab size). Each group will be responsible for exposing one organism to UV light for specific time points. Refer to Table 42.1 for your group assignment. ***Note:*** Two *Bacillus subtilis* cultures are used in this experiment, one is a fresh culture and the other is 1 week old. As a reminder, *Bacillus* species can form endospores when stressed. Older cultures are more likely to have undergone sporulation than fresh cultures. Therefore, as you perform the experiment and analyze the results, think about the susceptibility of these two cultures to UV-C treatments.

Clinical Applications

Ultraviolet light is useful for disinfecting laboratory and health-care environment work surfaces and surrounding air. It is also used to treat water and food packaging to reduce water and foodborne illnesses.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Short wavelength ultraviolet lamp (UV-C) with appropriate shielding and support
- ❑ Cardboard to cover plates (see Fig. 42.2)
- ❑ Tryptic soy agar (TSA) plates (4 per group; 8 for group 1)
- ❑ Stopwatch or electronic timer
- ❑ Sterile cotton swabs (4 per group; 8 for group 1)
- ❑ Fresh broth cultures of these recommended organisms (only 1 per group):
  - ❑ *Bacillus subtilis*
  - ❑ *Bacillus subtilis* 7-day culture
  - ❑ *Deinococcus radiophilus*
  - ❑ *Escherichia coli*

PROCEDURE

Today you will compare the effect of UV exposure on four cultures: *Bacillus subtilis* (24-hour and 7-day cultures), *Deinococcus radiophilus*, and *Escherichia coli*. Because of the large number of plates to be treated, the work will be divided among six groups of students. Refer to Table 42.1 for assignments. ***Note:*** If the class doesn't divide conveniently into six groups, then the groups assigned to the shorter exposure times can do more than one set of plates. During the lab exercise, you should wear your lab coat, eye protection, and gloves.

1. **Setting up:** Enter your group number and exposure time (from Table 42.1) on the data sheet at the end of this exercise. Then, obtain four TSA plates and label the bottom of each with the names of the organisms to be inoculated and your group number. Draw a line to divide the plates in half, and label the sides "A" and "B."
2. **Obtain Your Stock Culture:** Your group was assigned a specific bacterium to test. Obtain the stock culture and note the name of the organism here:

   _____
3. **Inoculate the Plates:** Dip a sterile cotton swab into the broth culture and wipe the excess on the inside of the tube so that the swab is not dripping wet. Inoculate the appropriate plate by spreading the organism over the entire surface of the agar. Do this by streaking the plate surface completely three times, rotating it by one-third between streaks. There should be no gaps between streaks. When incubated, this will form a bacterial lawn. Inoculate each of your four plates in this manner.

4 **Arranging Inoculated Plates for UV-C Treatment:** Per Figure 42.2, for each UV time point, you will arrange inoculated plates under the UV lamp with the agar surface exposed. Set the lids of each plate open side down on a disinfectant-soaked towel. You'll put the lids back on the plates after completing the UV treatment. Once you have all plates arranged, place a piece of cardboard over the plates so that Side B of each plate is shielded.

5 **Turn on the UV Lamp:** Be sure the UV lamp is the same distance from the plates for all exposures (we recommend between 8 and 12 inches). Then, turn on the lamp for the prescribed time. *Caution!* Be sure the protective shield is in place and do not look at the light while it is on!

6 **Incubate the Plates:** After the UV treatment is finished, turn off the UV light and immediately replace the lids for each plate. Then invert the plates and incubate them as follows:

- *B. subtilis* and *E. coli*, 35±2°C for 24 to 48 hours.
- *D. radiophilus*, 30°C for 72 to 96 hours.

7 **Repeat the Process:** Repeat the plate exposure process (Steps 4–6) for each UV treatment time point.

8 **Clean up:** Save or dispose of the stock cultures as your instructor directs, disinfect your work surface, and thoroughly wash your hands with soap and water before leaving the lab.

→ Next Lab Session

1 **Examine the Samples:** Remove your plates from the incubator and observe for growth. Side B should be covered with a bacterial lawn (confluent growth). If this is not the case, see your instructor.

2 **Record Your Observations:** Note the extent of growth on Side A of each plate. Record your observations in the table in the data sheet at the end of this exercise. So that groups can easily compare data, record the level of growth on each of your group's plates as "heavy confluent growth," "sparse confluent growth," "individual colonies," or "no growth."

3 **Record Data from Other Groups:** Your instructor will provide you with a means to share data obtained by all groups. Record these data on your data sheet at the end of this exercise.

4 **Graph the Data:** On the graph paper provided with the data sheet, construct a graph representing growth versus UV exposure time for all three organisms. If you need help with graphing, see pages 10–12.

5 **Clean up:** Dispose of the samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

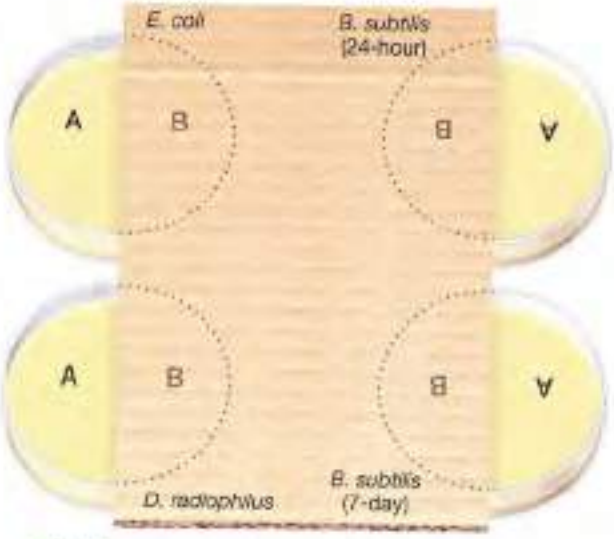


FIGURE **42.2** **Plates Shielded for UV Exposure** • Place the plates under the UV lamp with the lids removed and the cardboard shield covering half of each plate as shown. Make sure the petri plate lids are placed open side down on a disinfectant-soaked towel.

TABLE **42.1** Group Assignments •

Organism	No UV	1 Minute	2 Minutes	4 Minutes	8 Minutes	12 Minutes	16 Minutes
B. subtilis (24-hour culture)	1	1	2	3	4	5	6
B. subtilis (7-day culture)	1	1	2	3	4	5	6
Deinococcus radiophilus	1	1	2	3	4	5	6
E. coli	1	1	2	3	4	5	6

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 42

DATA SHEET

The Effect of Ultraviolet Radiation on Microbial Growth

During the Lab

1 Enter your class data in the table provided using the following descriptions:

0 = no growth

1 = individual colonies

2 = sparse confluent growth

3 = heavy confluent growth

UV Exposure (minutes)	*B. subtilis* (24-hour culture)		*B. subtilis* (7-day culture)		*D. radiophilus*		*E. coli*	
	Side A	Side B	Side A	Side B	Side A	Side B	Side A	Side B

Lab Related Questions

1 Why were you told to remove the plate covers prior to exposing them to UV?

a. If the lids were left on, the heat generated during the treatment would be trapped, which would cause the temperature of the cultures to increase to a level that would kill the bacteria on the plates. This temperature-induced killing would therefore eliminate perceivable differences among the treated plates.

b. If the lids were left on, they would block UV-C rays, thereby sparing all bacteria and eliminating perceivable differences among the treated plates.

c. If the lids were left on, they would intensify the UV-C rays, to the point of killing all the inoculated bacteria on the plates and thus a difference among the treatments would not be evident.

d. The removal of the lids was necessary to avoid differences in UV exposure due to variances in lid thickness.

e. If the lids had been left on, condensation would have formed on the inner lid surface and spilled back onto the cultures during the UV exposure, thereby contaminating the plates.

2 What is the general relationship between bacterial growth and UV exposure time?

a. As UV exposure time increases, growth decreases.
b. As UV exposure time increases, growth increases.
c. As UV exposure time decreases, growth decreases.
d. UV exposure time does not affect bacterial growth.

3 The purpose of this exercise is to demonstrate the comparative effect of UV on four bacterial populations. This could have been accomplished without the cardboard cover. What statement best explains why the cover was used?

a. It allowed you to conduct the experiments using fewer plates.
b. It reduced the time for conducting the experiment.
c. It enhanced the UV intensity for Side A because a smaller surface area was exposed to the light.
d. It allowed for side-by-side comparisons of an experimental and a control all on the same plate.
e. It allowed you to conduct the experiment in less time.

4 Assume the UV lamp is bumped and, as a result, it is located twice as far from the plates for the last time point (16 minutes) as compared to each of the earlier time points. What result would you least expect?

a. The time 0 plate would have more growth than the 16-minute plate.
b. The 1-minute plate would exhibit more growth than the 16-minute plate.
c. The 12-minute plate would exhibit less growth than the 16-minute plate.
d. The 12-minute plate would exhibit more growth than the 16-minute plate.

5 The intensity of a UV exposure _____ as the distance from the target decreases.

6 List the pros and cons of UV-C radiation as a means of achieving disinfection.

_____

_____

_____

_____

_____

7 The germicidal action of UV-C is primarily due to:

a. Damage to the bacterial cell wall.
b. Heat generated by the UV radiation.
c. Destruction of cellular proteins.
d. Interference with biochemical pathways.
e. Induced nucleic acid damage.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

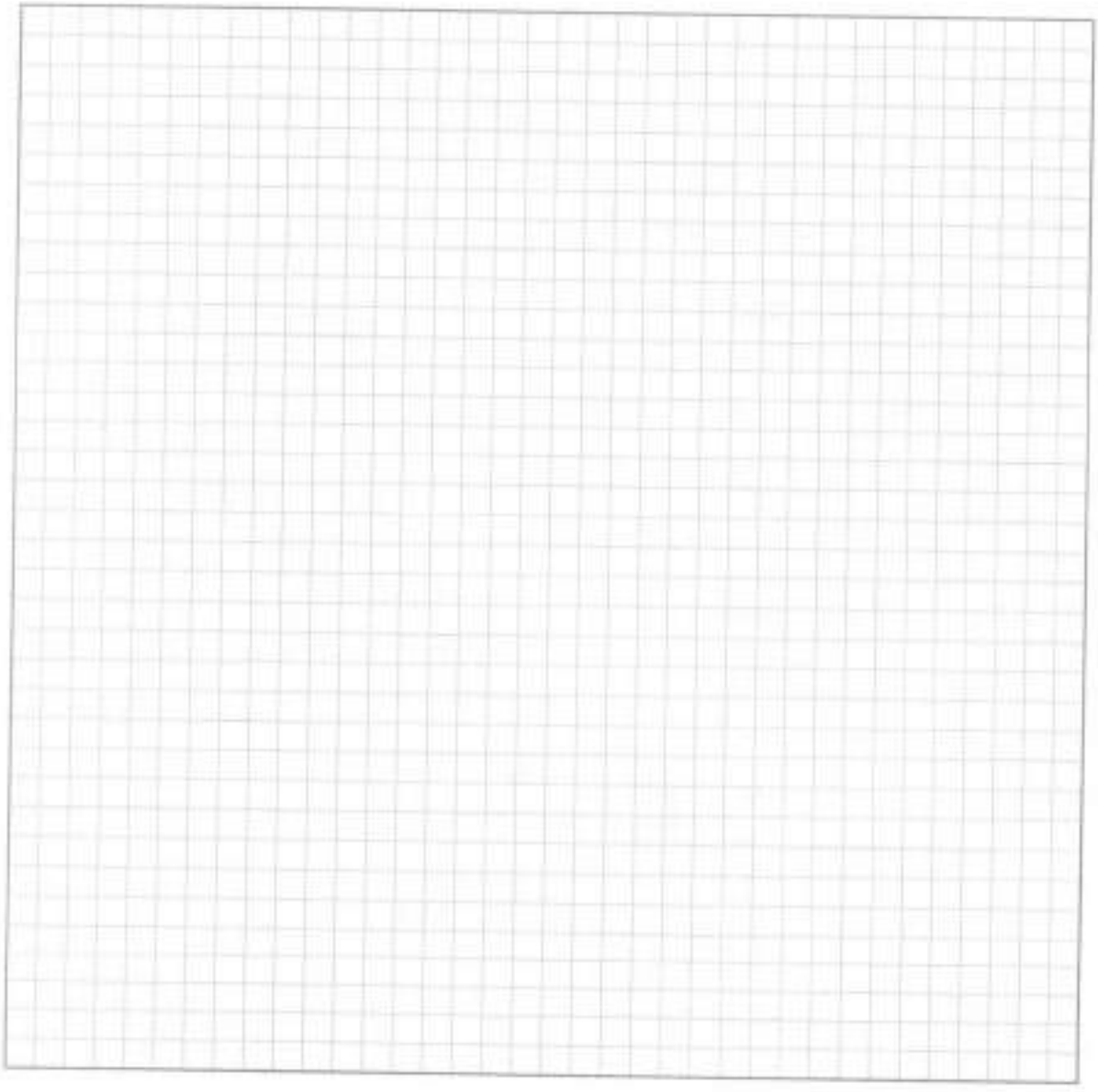
EXERCISE 42 DATA SHEET

(continued)

Critical Thinking

1 Which organism survived the longest exposure? Why do you suppose it did?

2 Using the graph paper provided, construct a single graph of relative growth (0, 1, 2, 3) versus UV exposure time for the four cultures. Include a legend defining the numbers and symbols/line colors for the different cultures. See pages 10–12 for proper graphing technique.



— Lab Notes —

EXERCISE 43

Antimicrobial Susceptibility Test: Disk Diffusion (Kirby-Bauer) Method

After doing this exercise, you should be able to:

1. Describe how the disk diffusion test for antimicrobial drug susceptibility testing works and what it reveals.
2. Define what a zone of inhibition is and describe how it is evaluated in the disk diffusion test.
3. State a key limitation of the disk diffusion test.
4. Define the terms *bactericidal* and *bacteriostatic*.
5. Explain the standardized aspects of the Kirby-Bauer procedure.
6. Describe what an antibiogram is and discuss why it is clinically useful.

Background

In this exercise, you will perform an antimicrobial susceptibility test known as the **disk diffusion (Kirby-Bauer) method.** This test is useful for assessing a pure culture's susceptibility to various antimicrobial drugs.[1] In 1966, Alfred Bauer, William Kirby, and associates first published a standardized disk diffusion method of testing the effectiveness of more than 20 antimicrobial agents. Their methodology, which bears their names, is a valuable tool for establishing the effectiveness of antimicrobics against pathogenic microorganisms in clinical laboratories.

In the test, antimicrobic-infused paper disks are placed on an inoculated agar plate (Fig. 43.1). Once the disks are in position, the plates are incubated at 35±2°C for 16 to 18 hours. During incubation, the bacteria grow as a **confluent lawn** (a uniform spread of bacterial growth on an agar plate's surface as opposed to streaks, patches, or colonies on the media's surface). Also, during incubation, the drugs infused into the paper disks diffuse into the agar. As each drug diffuses through the agar away from the disk, a concentration gradient develops in which the drug concentration *decreases* as the distance from the disk *increases*. Following incubation, a clear zone called a **zone of inhibition (ZOI)** may be visible around some of the disks. These clearings are areas where the antimicrobial drug concentration was high enough to inhibit bacterial growth (Fig. 43.2). The margin of the ZOI reflects the **minimum inhibitory concentration (MIC)** of the drug needed to interfere with bacterial growth. As performed in this exercise, we will not be able to quantify the MIC; however, there are modified versions of the diffusion test—namely, the **E-test**—that can quantify the MIC.

If a ZOI is *not* observed around a disk, then the bacterium is by default considered resistant to the drug. However, the mere presence of a ZOI does not

FIGURE **43.1 Disk Dispenser** ● A disk dispenser, like the one shown, is sometimes used to uniformly deposit disks on a Mueller-Hinton agar plate.

[1] Notice that here we refer to antimicrobial drugs rather than antibiotics. This is because the term *antibiotic* technically only refers to naturally occurring antimicrobial agents produced by microorganisms. For example, the mold *Penicillium notatum* naturally makes the antibiotic penicillin. Today many of the antibacterial agents that treat bacterial infections are synthetic, so the terms *antimicrobial*, *antimicrobic*, or *antibacterial* drug are arguably more appropriate ways of referring to these agents. That said, it's common for people (including many scientists and clinicians) to simply use the term *antibiotic* to refer to *all* antibacterial drugs (both natural and synthetic) as antibiotics. The main point is that you know the difference for an exam should your instructor highlight this point!

automatically mean the bacterium is susceptible to the drug that generated the ZOI. Instead, the diameter of the zone, which is measured in mm and includes the disk diameter, must be measured (Fig. 43.3) and compared to standardized criterion measurements to determine if the tested bacterium is resistant (R), susceptible (S), or intermediate (I) in its response to the given drug (Table 43.1).

It is important to appreciate that the cleared zone devoid of bacterial growth does not necessarily mean that the drug killed the bacteria in that area; you cannot assume the drug is **bactericidal**. The clear zone may have developed because the drug is **bacteriostatic** and only inhibiting the bacterium's growth. An important limitation of the disk diffusion (Kirby-Bauer) method is that it does not reveal if the drugs used in the test are bactericidal or bacteriostatic against the assessed bacterium. It is worth clarifying that from a clinical standpoint, bactericidal drugs are not necessarily considered superior to bacteriostatic drugs.

FIGURE **43.2** **Disk Diffusion Test of Methicillin-Resistant *Staphylococcus aureus* (MRSA)** • This plate illustrates the effect of (clockwise from top outer right) Nitrofurantoin (F/M300), Norfloxacin (NOR 10), Oxacillin (OX 1), Sulfisoxazole (G 0.25), Ticarcillin (TIC 75), Trimethoprim-Sulfamethoxazole (SXT), Tetracycline (TE 30), Ceftizoxime (ZOX 30), Ciprofloxacin (CIP 5), and (inner circle from right) Penicillin (P 10), Vancomycin (VA 30), and Trimethoprim (TMP 5) on Methicillin-resistant *Staphylococcus aureus* isolated from a patient. **Note:** This petri plate is 150 mm in diameter to accommodate 12 disks.

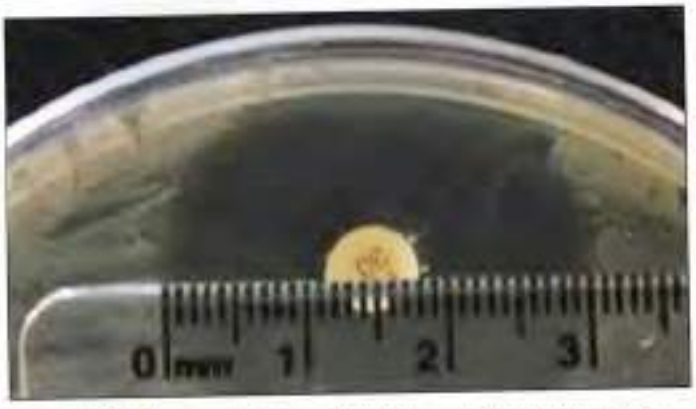


FIGURE **43.3** **Measuring Zones of Inhibition** • Using a metric ruler and a dark, nonreflective background, measure the diameter of each clearing in millimeters (mm). This zone is 29 mm in diameter.

Standardized Aspects of the Kirby-Bauer Procedure

All aspects of the Kirby-Bauer procedure are standardized to ensure reliable results. Therefore, care must be taken to adhere to these standards.

Agar pH and Depth

Mueller-Hinton agar pH is between 7.2 and 7.4. This pH must be strictly adhered to when making the media because fluctuations either above or below the stated pH levels can affect drug activity. Also, the agar is always poured to a depth of 4 mm; this is true regardless of the plate size used (e.g., 150 mm or 100 mm petri plates). The agar depth is important to standardize because it can affect drug diffusion. Thick agar slows lateral diffusion and thus produces smaller zones than plates held to the 4 mm standard. In contrast, if the agar is thinner than the 4 mm standard, then the drugs will diffuse further than normal and may lead to artificially larger ZOIs, which may lead us to inaccurately conclude the tested bacterium is susceptible to the given drug.

Sample Turbidity

Another aspect of the Kirby-Bauer procedure that's standardized is the density of the bacterial culture inoculated onto the media. Typically, the bacterial sample to be tested is grown as a broth culture that is incubated for 4 to 6 hours to bring the cells into the **exponential phase of growth**, or **logarithmic growth stage** (log stage), the stage at which the bacteria are typically the healthiest and actively dividing. Because many antibiotics target the cellular processes of actively growing cells, it's essential to have the cells in this phase of growth before testing their susceptibility to antibacterial drugs. Failure to do so may lead to inaccurate data regarding drug susceptibility. After the incubation period, the culture is subsequently diluted to match a 0.5 **McFarland turbidity standard** (Fig. 43.4). A McFarland Standard is a solution of barium chloride and sulfuric acid that react to form a fine precipitate of barium sulfate, which optically mimics a turbid bacterial sample. The 0.5 McFarland Standard corresponds to a cell density between 1 and 2×10^8 CFU/mL (colony forming units/mL). An alternative to the McFarland approach is to use a spectrophotometer

TABLE 43.1 Zone Diameter Interpretive Chart ●

Antibiotic	Organism(s)	Code	Disk Potency	Zone Diameter Interpretive Standards (mm)		
				Resistant	Intermediate	Susceptible
Chloramphenicol	*Enterobacteriaceae* and *Staphylococcus*	C 30	30 µg	≤ 12	13–17	≥ 18
Ciprofloxacin	*Enterobacteriaceae* and *Staphylococcus*	CIP 5	5 µg	≤ 15	16–20	≥ 21
Penicillin	*Staphylococcus*	P 10	10 U	≤ 28		≥ 29
Streptomycin	*Enterobacteriaceae*	S 10	10 µg	≤ 11	12–14	≥ 15
Tetracycline	*Enterobacteriaceae* *Staphylococcus*	TE 30 TE 30	30 µg 30 µg	≤ 11 ≤ 14	12–14 15–18	≥ 15 ≥ 19
Trimethoprim	*Enterobacteriaceae* and *Staphylococcus*	TMP 5	5 µg	≤ 10	11–15	≥ 16

This table includes the antibiotics used in this exercise and contains data provided by the Clinical and Laboratory Standards Institute (CLSI). Permission to use portions (specifically Tables 2A and 2C) of *M100 Performance Standards for Antimicrobial Susceptibility Testing, 30th edition* (2020) has been granted by CLSI. The interpretive data are only valid if the methodology in CLSI Standard *M02 Performance Standards for Antimicrobial Disk Susceptibility Tests*, 13th edition, (2018) is followed. CLSI updates the interpretive tables in M100 annually through new editions of the supplement. Users should refer to the most recent editions. The most current editions of both documents may be obtained from CLSI, 950 West Valley Road, Suite 2500, Wayne, PA 19087. Contact also may be made via the website (www.CLSI.org), email (customerservice@clsi.org), and by phone (1.877.447.1888).

to bring the broth culture to an absorbance of 0.08 to 0.10 at 625 nm in a 1 cm cuvette. This absorbance corresponds to the 0.5 McFarland standard.

FIGURE 43.4 McFarland Standards ● This image shows a comparison of a 0.5 McFarland turbidity standard to three broths of varying turbidity. A visual comparison of turbidity is made by placing a card with sharp black lines behind the tubes. Notice that Tube 2's turbidity matches the McFarland standard exactly, whereas Tube 1 is too turbid and Tube 3 is too clear.

Number and Spacing of Disks on a Plate

The number of disks used on a given plate depends on the plate size. For 150 mm plates, place no more than 12 disks on the agar surface. For 100 mm plates, the size of the plates used in this exercise, a maximum of 6 disks should be used. The positioning of the drug-infused disks is also standardized. There must be a spacing of at least 24 mm between disks (as measured from one disk's center to the adjacent disk's center).

Drug Concentrations and Interpretive Standards

The U.S. Food and Drug Administration establishes the disk concentration of each agent, which is printed on the disk along with a code indicating the chemical agent. The Clinical Laboratory Standards Institute (CLSI) in Wayne, Pennsylvania, has performed extensive research (and continues to do so) to establish the ZOI diameter interpretive standards like those presented in Table 43.1. ***Note:*** Many more antimicrobial agent/pathogen combinations exist than are represented in the provided table. Instead, the full collection of data is housed in a resource called the *M100 Performance Standards for Antimicrobial Susceptibility Testing*. This reference is annually updated and is the source of the information in Table 43.1.

The CLSI establishes the interpretive criteria regarding zone diameter breakpoints for resistance and susceptibility. The cutoff criteria are based on zone of inhibition diameters obtained for bacteria known to be susceptible to the given drug. If an observed ZOI's diameter is *less* than the ZOI diameter obtained for related susceptible bacterium, then the ZOI diameter is said to fall within the **resistance breakpoint**. This means the tested bacterium grew even when exposed to a higher drug concentration than that which inhibits the growth of a related bacterium known to be susceptible to the drug. Likewise, the ZOI diameter above which all susceptible strains fall is the **susceptibility breakpoint**. In some cases, there may be an intermediate range of zone diameters in which some resistant and susceptible strains fall.

A Note on Bacterial Susceptibility to Antimicrobial Drugs

Bacterial resistance to antimicrobial drugs is a complex matter, and increasingly problematic in health care. Although a full discussion of drug resistance mechanisms is beyond the scope of this exercise, you're encouraged to review Table 43.2, which presents the modes of drug action for the drugs used in this exercise and examples of how bacteria may develop resistance to the tested drugs.

Clinical Applications

The disk diffusion test is a standardized method used to assess if microorganisms are susceptible to specific antimicrobial drugs. Ultimately, susceptibility testing provides important information that helps clinicians decide which antimicrobial drug(s) would be best for treating a patient's infection.

To improve patient care, clinical microbiology labs produce drug susceptibility reports called **antibiograms**. An antibiogram can be based on a specific isolate for a single patient sample, or it can be a broader report (e.g., institutional antibiogram) that conveys drug susceptibility data for bacterial species isolated from patients at a specific health-care facility (e.g., hospital, long-term care center, etc.). Because bacteria are constantly evolving drug resistance, CLSI recommends that health-care facilities update their institutional antibiogram data at least once per year. This helps to ensure that clinicians have accurate data regarding the usefulness of specific antimicrobial drugs against bacterial strains circulating in the given health-care setting. For example, if a clinician knows their patient has an *E. coli* infection and the hospital's most recent antibiogram shows most *E. coli* isolates from patients in the hospital are resistant to a

TABLE **43.2** Antibiotic Targets and Resistance Mechanisms •

Antibacterial Drug	Cellular Target	Resistance Mechanism
Chloramphenicol	Prevents peptide bond formation during translation	1. Poor uptake of drug 2. Inactivation of drug
Ciprofloxacin	Interferes with DNA replication	1. Altered target 2. Poor uptake of drug
Penicillin	Inhibits cross-linking of the cell wall's peptidoglycan	One or more of: 1. Altered target 2. Poor uptake of drug 3. Production of β-lactamases
Streptomycin	Blocks initiation complex formation in protein synthesis	1. Altered target
Tetracycline	Blocks attachment of aminoacyl tRNA to A site on ribosome	1. Efflux mechanism
Trimethoprim	Inhibits purine and pyrimidine synthesis	1. Altered target

Not all antimicrobial drugs affect cells in the same way. Some attack the bacterial cell wall, and others interfere with biosynthesis reactions. There are three main drug resistance mechanisms: (a) **target alteration:** the cell may alter the drug's target such that the antibacterial drug is less effective at interacting with its target; (b) **drug inactivation:** the cell may break down or alter the antibacterial drug rendering it less effective; and (c) **reducing the drug's concentration inside the cell:** a cell can limit or block drug entry or even pump the drug out of the cell via efflux pumps.

particular drug—say, tobramycin—then the clinician would avoid prescribing tobramycin and would instead select a more effective drug.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ 100 mm Mueller-Hinton agar plates (2)
- ❑ Penicillin, chloramphenicol, tetracycline (optional), trimethoprim, streptomycin (optional), and ciprofloxacin antibiotic disks (and/or other disks as available)
- ❑ Antibiotic disk dispenser or forceps for disk placement
- ❑ Screw-capped jar of alcohol (for sterilizing forceps, if used)
- ❑ Sterile cotton-tipped swabs (2)
- ❑ Metric ruler (1)
- ❑ Sterile saline (0.85%)
- ❑ Sterile transfer pipettes (2)
- ❑ McFarland 0.5 standard with card (1)
- ❑ Black, nonreflective poster board (8.5" × 11")
- ❑ Fresh broth cultures of these recommended organisms:
  - ☐ *Escherichia coli*
  - ☐ *Staphylococcus aureus* (BSL-2)
  - ☐ *Staphylococcus epidermidis* (a BSL-1 substitute for *S. aureus*)

Optional Materials (necessary if not using McFarland standard)

- ❑ Spectrophotometer
- ❑ Matched, sterile, 1 cm cuvettes (3)
- ❑ Sterile transfer pipettes (3)
- ❑ Capped, sterile 12 mm test tubes (2); and 75 mm test tubes (2)
- ❑ Tryptic soy agar (TSA) (or nutrient agar, NA) plates (2; for optional procedure)

Media Recipe

Mueller-Hinton II Agar (MHA)

Beef extract	2.0 g
Acid hydrolysate of casein	17.5 g
Starch	1.5 g
Agar	17.0 g
Distilled or deionized water	1.0 L

Adjusted to pH 7.2–7.4 at 25°C

PROCEDURE

You will test the susceptibility of *Escherichia coli* and either *Staphylococcus aureus* or *S. epidermidis* to penicillin, chloramphenicol, tetracycline (optional), trimethoprim, streptomycin (optional), and ciprofloxacin. These antimicrobial drugs were chosen because they exhibit different modes of action on bacterial cells (Table 43.1). Wear your lab coat, eye protection, and gloves throughout the procedure. ***Caution!*** Follow BSL-2 precautions if using *S. aureus*.

1. **Obtain the Stock Cultures and MHA Media:** Gently mix the stock cultures until they reach their maximum turbidity. You'll also need two plates of MHA media. Label the bottom of each MHA plate with your name and the date. Then, add the label "*E. coli*" to one plate and designate the second one as "*S. aureus*" or "*S. epidermidis*," depending on which bacteria your instructor has available for your lab session.

2. **Check the Stock Culture Turbidity:** In some cases, your instructor may provide prediluted stock bacterial samples that are ready for you to inoculate. If that is the case, you may skip to Step 3. However, if your instructor does *not* provide you with samples that are prediluted to the proper turbidity, you must adjust the sample concentrations before inoculating the media. Following are two methods for doing this. Use the method your instructor selects.

   - ***McFarland Method:*** Holding the culture and McFarland standard upright in front of you, place the card behind them so you can see the black line(s) through the liquid in the tubes. As you can see in Figure 43.4, the line becomes distorted by the sample turbidity. Use the black line to compare the turbidity level of the two tubes. Dilute the broth with sterile saline until it appears to have the same turbidity as the standard. If it is not turbid enough, incubate it until it reaches that level. Repeat this process for all bacterial cultures you will use in this exercise.
   - ***Spectrophotometer Method:*** Rather than using the McFarland approach, your instructor may prefer that you use a spectrophotometer to bring the samples to the proper growth density. To do this, you need to first prepare a "blank sample" to zero out the spectrophotometer before you read your stock bacterial sample turbidity. You can set up your blank sample by using a sterile transfer pipette to transfer some sterile broth (the same as that used to grow the bacterial stock cultures) into a cuvette. Then, follow your instructor's directions regarding how to "zero out," or blank, the spectrophotometer in your lab. Next, mix the *E. coli* culture and use a sterile pipette to aseptically transfer some of the stock culture into a

second cuvette. You should determine the sample absorbance at 625 nm. Depending upon the reading, either dilute the stock culture or let it grow longer to bring its absorbance to 0.08 to 0.10. Repeat this process for all bacterial cultures you will use in this exercise.

3 **Inoculate the Medium with *E. coli*:** Dip a sterile swab into the *E. coli* stock culture that is at the correct turbidity. As you remove the swab, press and rotate the cotton tip against the side of the tube to remove excess broth so that the swab is not dripping wet. Then, use the swab to streak the entire surface of the agar. Your goal is to achieve a confluent lawn of growth, so make the streaks right next to each other and cover the entire surface of the media. ***Caution!*** To avoid contamination, use the lid to guard your plate's surface. Never take the lid completely off and never set the lid on the bench surface!

To ensure full coverage, once you have streaked the entire plate surface, you should rotate the plate by one-third and repeat the process with the same swab (do *not* redip the swab in the culture). Once you've completed this second pass, you'll perform a third and final streaking (do *not* redip the swab in the culture) by again rotating the plate by one-third and then restreaking the media's entire surface. When finished, properly dispose of the swab and replace the plate's lid.

4 **Inoculate the Medium with *S. aureus* or *S. epidermidis*:** Repeat Step 3, but this time use the *S. aureus* or *S. epidermidis* culture.

5 **Apply Disks to the Media:** Apply penicillin-, chloramphenicol-, ciprofloxacin-, and trimethoprim-infused disks to the agar surface of each plate. If you do not have a disk dispenser, you can apply the disks singly using alcohol-flamed forceps[2]. Be sure to space the disks sufficiently apart (at least 24 mm between disks) to prevent zones of inhibition from overlapping. You also must keep the disks away from the plate edge. Using your forceps, gently tap the disks so they make uniform contact with the agar surface, but do *not* push the disks into the agar.

6 **Incubate Your Plates:** Incubate your inverted plates aerobically at 35±2°C for 16 to 18 hours.

7 **Clean up:** Dispose of the materials as your instructor directs, disinfect your work surface, and thoroughly wash your hands with soap and water before leaving the lab.

→ Next Lab Session

1 **Examine the Samples:** Remove the plates from the incubator (or refrigerator). Hold each plate over the black, nonreflective poster board and examine the plate with reflected light. The edge of a zone is where no growth is visible to the naked eye. Measure the diameter of each zone of inhibition in millimeters (Fig. 43.3).

2 **Record Your Observations:** Use Table 43.1 to help you record your results in the data table at the end of this exercise. If you used additional antibiotics, then refer to the ZOI diameter data that the manufacturer includes with all antibiotic disks.

3 **Clean up:** Dispose of the plates as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

Optional Procedure

You would start this optional procedure on the same day that you make your ZOI observations. This procedure is to examine if the drugs that generated ZOIs were bacteriostatic or bactericidal in their action toward the tested bacteria. Wear a lab coat, gloves, and eye protection when performing this procedure. ***Caution!*** Follow BSL-2 precautions if using *S. aureus*.

1 **Obtain and Label TSA or NA Media:** Obtain two TSA or NA plates. With your marking pen, divide the plates into four sectors (or more if you used more antibiotics). Label both plates with your group name. Label one plate *E. coli* and the other *S. aureus* (or *S. epidermidis*). Label each sector with the name of the antibiotic that generated a ZOI. It is easiest if you label your plate in the same order as the drugs are placed on your MHA plates.

2 **Inoculate the Media:** Using a sterile loop for each transfer, obtain a sample from each antibiotic's zone of inhibition on the *E. coli* plate, and spot-inoculate the corresponding sector on the TSA (or NA) plate. If there is no zone for a particular antibiotic, no transfer is necessary. Repeat with the *S. aureus* (or *S. epidermidis*) MHA plate.

[2] Keep the forceps in the alcohol jar until ready to use them. Then, close them as you remove them from the alcohol. This will cop some alcohol between the tips. Pass the forceps *through* the flame and allow the alcohol to burn off *away from the alcohol jar and the flame*. When the flame burns out, give the forceps a few seconds to cool, and then place the disk on the plate. Return the forceps to the alcohol jar until needed. *Caution!* Keep the flaming forceps away from the alcohol jar. If the alcohol in the jar catches fire, extinguish it by replacing the jar's lid.

3 **Incubate Your Plates:** Incubate the inverted plates aerobically at 35±2° for 24–48 hours.

4 **Clean up:** Dispose of the materials as your instructor directs, disinfect your work surface, and thoroughly wash your hands with soap and water before leaving the lab.

→ Next Lab Session

1 **Examine the Samples:** Remove the plates from the incubator and examine each sector for growth.

2 **Record Your Observations:** Record your observations and answer the questions on the data sheet provided at the end of this exercise.

3 **Clean up:** Dispose of the materials as your instructor directs, disinfect your work surface, and thoroughly wash your hands with soap and water before leaving the lab.

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 43

DATA SHEET

Antimicrobial Susceptibility Test: Disk Diffusion (Kirby-Bauer) Method

During the Lab

1 Record the zone of inhibition diameters (mm) in the data table provided. Then, use Table 43.1 as a guide to assess if the bacterium was susceptible (S), resistant (R), or intermediate (I) in its response to the tested antimicrobics. (Not all combinations of antibiotics and organisms are presented in Table 43.1. If you used antimicrobics not listed, then refer to the ZOI data that the manufacturer includes with all antibiotic disks.)

Interpreting Zone of Inhibition (ZOI) Diameter Data

Organism	Chloramphenicol		Ciprofloxacin		Trimethoprim		Penicillin		Streptomycin (optional)		Tetracycline (optional)	
	Zone Diameter	S/R	Zone Diameter	S/R	Zone Diameter	S/R	Zone Diameter	S/R	Zone Diameter	S/R	Zone Diameter	S/R
E. coli												
S. aureus (or *S. epidermidis*)												

2 If you did the optional exercise, record "G" for growth and "NG" for no growth in the data table provided. Then, record your conclusion regarding the antimicrobial drug's bactericidal or bacteriostatic effects on the tested bacteria.

Bactericidal or Bacteriostatic Effects on Test Bacteria

Organism	Chloramphenicol		Ciprofloxacin		Trimethoprim		Penicillin		Streptomycin (optional)		Tetracycline (optional)	
	NG/G	-static or -cidal	NG/G	-static or -cidal	NG/G	-static or -cidal	NG/G	-static or -cidal	NG/G	-static or -cidal	NG/G	-static or -cidal
E. coli												
S. aureus (or *S. epidermidis*)												

Lab Related Questions

1 Using Table 43.1, designate the following as resistant (R), intermediate (I), or susceptible (S).

_____ A *Staphylococcus* species with a ZOI of 20 mm around a ciprofloxacin disk.

_____ An *Enterobacteriaceae* species with a ZOI of 18 mm around a chloramphenicol disk.

_____ A *Staphylococcus* species with a ZOI of 10 mm around a trimethoprim disk.

_____ An *Enterobacteriaceae* species with a ZOI of 15 mm around a tetracycline disk.

_____ A *Staphylococcus* species with a ZOI of 27 mm around a penicillin disk.

2 Which of the following is (are) standardized in the disk diffusion test? (Select all that apply.)

a. The type of media used.
b. The depth of the media.
c. The concentration of the bacterial culture used for inoculation.
d. The size of the antimicrobial infused disks
e. The type of bacteria used.

3 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ TSA media is used to perform the Kirby-Bauer test.

_____ The Kirby-Bauer test does not reveal if a drug acts against an isolate in a bactericidal manner.

_____ Bacteria that lack a ZOI are automatically considered resistant to the tested antimicrobial drug.

_____ In the Kirby-Bauer test performed in this exercise, you used either a 1.0 McFarland standard or a spectrophotometer to assess culture density prior to inoculation.

4 Select the true statement(s) (select all that apply).

a. An institutional antibiogram for Hospital A in New York is usable at Hospital B in Colorado.
b. An antibiogram reveals which drugs are most effective in patients with specific underlying conditions.
c. An institutional antibiogram reveals which antimicrobial drugs are most likely to be effective against select species at a specific health-care facility.
d. CLSI recommend that institutions update their antibiograms at least every other year.
e. An antibiogram can be generated for a bacterium isolated from a specific patient sample.

5 Imagine you performed the exercise using MHA plates that were poured 2 mm deep instead of 4 mm deep. What is the most likely experimental outcome?

a. The ZOIs will have an artificially decreased diameter.
b. The ZOIs will have an artificially increased diameter.
c. The drugs will not diffuse from the disks into the agar.
d. The bacteria will not grow.
e. ZOIs will not develop.

6 Select the true statement.

a. The smaller a ZOI, the more concentrated a drug must be to prevent bacterial growth.
b. The smaller a ZOI, the less concentrated a drug must be to prevent bacterial growth.
c. The larger a ZOI, the more concentrated a drug must be to prevent bacterial growth.
d. ZOI diameter is not related to drug concentration; tested drugs are uniformly concentrated throughout a given ZOI.
e. Bactericidal drugs will generate larger ZOIs than bacteriostatic drugs.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 43 DATA SHEET

(continued)

7 In clinical applications of the Kirby-Bauer test, diluted cultures (for the McFarland standard comparison) must be used within 30 minutes. Why is this important?

a. If you delayed inoculation past 30 minutes, the bacterial growth will decrease due to nutrient limitation rendering the data inaccurate.

b. If you delayed inoculation past 30 minutes, the bacterial growth will develop resistance to the drugs in the media rendering the data inaccurate.

c. If you delayed inoculation past 30 minutes, the McFarland standard's density will be inaccurate.

d. If you delayed inoculation past 30 minutes, the bacterial growth will be too dense, and the data may therefore be inaccurate.

e. If you inoculate within 30 minutes, the bacterial growth will be too dense rendering the data inaccurate.

8 Was there a general difference in susceptibility between the tested Gram-positive bacterium (*S. aureus* or *S. epidermidis*) and the tested Gram-negative bacterium (*E. coli*)? If so, what difference(s) did you see?

Questions Related to the Optional Exercise

1 In the optional exercise, why were samples taken from the zones of inhibition for subculturing on TSA or NA plates?

a. To determine if the tested bacteria were susceptible to the tested drug(s).

b. To determine if the tested bacterial samples were contaminated.

c. To determine if the tested drug(s) killed or inhibited the bacteria from growing.

d. To determine if the tested bacteria can grow in the absence of the tested drug.

e. To determine if the tested bacteria are pathogens.

2 In this optional experiment, assume you obtained the following data:

Bacterium	Penicillin	Tetracycline	Ciprofloxacin
E. coli	Growth	Growth	No growth
S. epidermidis	No growth	No growth	Growth

Based on the data, what is the best conclusion?

a. Penicillin and tetracycline are more likely to kill *S. epidermidis* than *E. coli*.

b. *E. coli* is susceptible to ciprofloxacin whereas *S. epidermidis* is resistant to ciprofloxacin.

c. The tested drugs are more likely to kill *E. coli* than *S. epidermidis*.

d. Penicillin and tetracycline are bacteriostatic toward *S. epidermidis*.

e. Penicillin has bactericidal effects on *E. coli* and bacteriostatic effects on *S. epidermidis*.

Critical Thinking

The following is an excerpt from a hypothetical hospital antibiogram. Using the data in the antibiogram, answer the following questions.

	E. coli	*K. pneumoniae*	*P. mirabilis*	*P. aeruginosa*	*E. faecalis*	*S. aureus*	*S. epidermidis*	*S. pneumoniae*
	Gram-Negative Bacteria				**Gram-Positive Bacteria**			
Number of Isolates Analyzed	1654	98	142	190	398	350	202	51
Percent of Isolates Suceptible								
Ampicillin	51%	10%	94%	5%	98%	0%	0%	0%
Cefazolin	92%	99%	70%	0%	0%	99%	35%	0%
Levofloxacin	81%	97%	83%	79%	80%	75%	50%	98%

1 Your patient has a urinary tract infection caused by *E. coli* and wound infections caused by *S. aureus* and *P. aeruginosa*. Which antimicrobial drug(s) would you recommend for treatment and why? (**Note:** Based on patient history, none of the listed drugs are contraindicated and you can assume for this question that drugs can be prescribed alone or in any combination.)

2 Of the three drugs in the antibiogram, which one has the broadest activity against Gram-positive and Gram-negative bacteria? Why do you suppose it is so broadly effective against diverse Gram-positive and Gram-negative bacteria? (**Hint:** Consider how the drug you selected works.)

EXERCISE 44

Bacitracin, Novobiocin, and Optochin Susceptibility Tests

After doing this exercise, you should be able to:

1. Compare and contrast the procedure for assessing bacitracin, novobiocin, and optochin susceptibility to the Kirby-Bauer procedure.
2. Describe how bacitracin, novobiocin, and optochin susceptibility testing works and what these tests reveal.
3. Discuss the features of bacitracin, novobiocin, and optochin.
4. Describe what a zone of inhibition is and explain how it relates to assessing a bacterium's susceptibility to bacitracin, novobiocin, and/or optochin.
5. State how assessing bacitracin, novobiocin, and optochin susceptibility is clinically useful.

Background

In this exercise, you will assess bacterial susceptibility to three antimicrobial agents: bacitracin, novobiocin, and optochin. Although these susceptibility tests resemble the Kirby-Bauer procedure (disk diffusion test) reviewed in Exercise 43, they are far less rigorously standardized. The less stringent assessment parameters relate to the point that bacitracin, novobiocin, and optochin susceptibility tests are bacterial identification strategies. Unlike the Kirby-Bauer test, they are not for guiding decisions related to which drug is best for treating a patient infection.

As opposed to the Mueller-Hinton agar used in the Kirby-Bauer procedure, bacitracin, novobiocin, and optochin susceptibility testing procedures usually call for sheep blood agar so that hemolysis activity (Ex. 25) and drug susceptibility of an isolate can be assessed at the same time.[1] Similar to the Kirby-Bauer procedure, you will apply paper disks infused with a specific concentration of an antimicrobial substance onto confluently inoculated plates.[2] Then, following incubation, you will measure the diameter of resulting clear zones (zones of inhibition, or ZOIs) and compare your measurements to a standardized chart. Based on ZOI diameter, you'll decide if the tested bacterium is resistant or susceptible to the tested antimicrobial compound. Unlike the Kirby-Bauer procedure, an intermediate ranking (I) is not applicable in the bacitracin, novobiocin, and optochin susceptibility tests, nor are the cultures assessed for cell density before inoculation.

[1] Blood agar is relatively expensive, so your instructor may have you use TSA (tryptic soy agar) as a blood agar substitute. Such a substitution is acceptable in this exercise because you are using known bacteria and can therefore look up the hemolytic properties of each bacterium.

[2] Recall from Exercise 43 that a confluent inoculation generates a uniform spread of bacterial growth.

A Review of Bacitracin, Novobiocin, and Optochin

Each of the three agents in this exercise are active against certain bacteria. Bacitracin and novobiocin are both classified as antibiotics (compounds naturally made by other microbes) whereas optochin is not naturally occurring and is therefore considered an antimicrobial drug (or antimicrobic) rather than an antibiotic. Before performing the exercise, it's worth exploring a bit more about each of the three agents you will test.

Bacitracin, produced by *Bacillus licheniformis*, is a powerful peptide antibiotic that inhibits bacterial cell wall synthesis by interfering with transport of peptidoglycan subunits across the cytoplasmic membrane. Thus, it is only effective on actively growing bacteria that have a cell wall. If a zone of inhibition 10 mm or greater develops around a 0.04 U bacitracin disk (called an "A" disk), then the bacterium is bacitracin susceptible (Fig. 44.1). Bacitracin is a common active ingredient in certain over-the-counter topical antibacterial ointments (ointments applied to the skin's surface) such as Neosporin; however, it is rarely used in non-topical medications because when ingested or injected, it is associated with renal failure (kidney failure). Consequently, assessing bacitracin susceptibility is useful for bacterial identification, not to inform decisions about using bacitracin as a therapeutic.

Novobiocin is an antibiotic, produced by *Streptomyces niveus, a bacterium found in soil.* It is chemically related to coumarins, drugs that block blood clotting. Novobiocin interferes with ATPase activity associated with DNA gyrase, an enzyme necessary for DNA replication. A zone of inhibition 16 mm or more around a 5-µg disk is interpreted as novobiocin susceptibility (Fig. 44.2). As with bacitracin, novobiocin has toxic side effects in humans when ingested or injected. Consequently, it is not useful as a medication for humans, although it is occasionally used in veterinary medicine for treating larger animals.

Optochin is an antimicrobial substance derived from quinine. It targets the enzyme ATP synthase and thereby disrupts ATP production. A zone of inhibition 14 mm or more around a 6 mm optochin disk (called a "P" disk) containing 5 µg of optochin is interpreted as optochin susceptibility (Fig. 44.3). In the early 1900s, optochin was developed to combat bacterial pneumonia in humans, but because of its toxicity, it was never approved as a therapeutic.

FIGURE **44.1** **Bacitracin Susceptibility Test on a Sheep Blood Agar Plate** ● The zone diameter cut off for bacitracin susceptibility is 10 mm or more. The organism above has no clear zone and is resistant (R). The organism below has a zone diameter of 19 mm and is susceptible (S). The disks themselves are usually 6 mm and are referred to as "A" disks.

FIGURE **44.2** **Novobiocin Susceptibility Test on a Sheep Blood Agar Plate** ● The zone diameter cut off for novobiocin susceptibility is 16 mm or more. A novobiocin-resistant organism (R) with a zone diameter of 7 mm is on the left; a susceptible organism (S) with a zone diameter of 27 mm is on the right.

FIGURE **44.3** **Optochin Susceptibility Test on a Sheep Blood Agar Plate** ● The zone diameter cut off for optochin susceptibility is 14 mm or more. The zone of inhibition surrounding the "P" disk is 19 mm, indicating susceptibility to optochin. The α-hemolysis coupled with optochin susceptibility leads to presumptive identification of *S. pneumoniae*.

Clinical Applications

Unlike the Kirby-Bauer test, which helps clinicians decide which antimicrobial drug may be most effective against an infectious agent, the susceptibility tests performed in this exercise are used for bacterial identification.

The bacitracin test allows for a presumptive identification of the causative agent of strep throat (streptococcal pharyngitis), the β-hemolytic *Streptococcus pyogenes*. This pathogen is a Group A streptococcus that is bacitracin susceptible and thereby differentiated from other β-hemolytic streptococci that are bacitracin resistant. The bacitracin susceptibility test also differentiates members of the genus *Staphylococcus*, which are almost always resistant to bacitracin, from typically susceptible members of the genus *Micrococcus*. Recall that certain staphylococcus members, such as *S. aureus*, cause a variety of infections but are especially infamous for causing skin infections.

The novobiocin test differentiates coagulase-negative staphylococci (see Ex. 38). Most frequently it presumptively identifies *Staphylococcus saprophyticus*, which is novobiocin resistant. *S. saprophyticus* is a common cause of urinary tract infections.

The optochin test presumptively differentiates a common cause of pneumonia, *Streptococcus pneumoniae* (susceptible), from other α-hemolytic streptococci.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Blood agar plate (1 per test) (commercial preparation of TSA containing 5% sheep blood; TSA may be substituted for blood agar)
- ❑ Sterile cotton-tipped swabs (1 per organism to be tested)
- ❑ Screw-cap jar of alcohol with forceps

Bacitracin Test

- ❑ 0.04 unit bacitracin disks ("A" disks)
- ❑ Fresh broth cultures (18–24 hours old) of these recommended organisms:
  - ☐ *Staphylococcus aureus* (BSL-2)
  - ☐ *Micrococcus luteus*

Novobiocin Test

- ❑ 5 µg novobiocin disks
- ❑ Fresh broth cultures (18–24 hours old) of these recommended organisms:
  - ☐ *Staphylococcus epidermidis*
  - ☐ *Staphylococcus saprophyticus*

Optochin Test

- ❑ 5 µg (6 mm) optochin disks ("P" disks)
- ❑ Fresh broth cultures (18–24 hours old) of these recommended organisms:
  - ☐ *Streptococcus pneumoniae* (BSL-2)
  - ☐ *Streptococcus agalactiae* (BSL-2)

PROCEDURE

In this exercise, you will assess the susceptibility of test bacteria to one or more of the following: bacitracin, novobiocin, and optochin. Usually blood agar is used for these tests; however, for convenience and cost savings, TSA may be substituted in this lab. After inoculating the plates in a fashion that will generate a lawn of bacterial growth, you will place specific antibiotic disks in the center of the inoculum. Following incubation, you will measure the diameter of any resulting clear zones surrounding the disks and compare your findings to Tables 44.1, 44.2, and 44.3 to determine if your sample bacteria are susceptible or resistant to the tested compounds. Wear a lab coat, gloves, and eye protection when performing this procedure. **Use BSL-2 precautions as appropriate** for the organisms assigned. ***Caution!*** Follow BSL-2 precautions if using *S. aureus*, *S. pneumoniae*, or *S. agalactiae*.

TABLE 44.1 Bacitracin Test Results and Interpretations

Result	Interpretation	Symbol
Zone of clearing 10 mm or greater	Organism is susceptible to bacitracin; probable *Streptococcus pyogenes* if a β-hemolytic streptococcus, or *Micrococcus* species if a Gram-positive coccus	S
Zone of clearing less than 10 mm	Organism is resistant to bacitracin; not *Streptococcus pyogenes*; probable *Staphylococcus aureus* if Gram-positive coccus	R

TABLE 44.2 Novobiocin Test Results and Interpretations

Result	Interpretation	Symbol
Zone of clearing 16 mm or greater	Organism is susceptible to novobiocin; not likely *Staphylococcus saprophyticus* if a coagulase-negative staphylococcus	S
Zone of clearing less than 16 mm	Organism is resistant to novobiocin; probable *Staphylococcus saprophyticus* if a coagulase-negative staphylococcus	R

TABLE 44.3 Optochin Test Results and Interpretations

Result	Interpretation	Symbol
Zone of clearing 14 mm or greater	Organism is susceptible to optochin; probable *Streptococcus pneumoniae* if an α-hemolytic streptococcus	S
Zone of clearing less than 14 mm	Organism is resistant to optochin; not *Streptococcus pneumoniae* if an α-hemolytic streptococcus	R

1 Obtain and Label the Plated Media: For each drug being tested, you will need one TSA or blood agar plate and two test bacteria. Label the bottom of each plate with your name and the date. Then draw a line down the middle of the plate and label one half of the plate with the name of one of the test bacteria, and the other half with the name of the other bacterium. Make sure you have the correct organisms for each antimicrobial drug you were told to test!

2 **Inoculate Half of the Plate:** Gently mix the stock culture. Avoid splashing it into the tube's cap. Then, dip a sterile cotton-tipped swab into the bacterial stock. Before removing the swab, press the cotton tip against the inside of the tube to squeeze out excess fluid so that the swab is not dripping wet when removed. Inoculate the plate by making back-and-forth streaks that stop short of the line you drew on the bottom of the plate. Then, turn the plate and evenly run the swab over the same half of the plate in strokes perpendicular to those you previously made. Your goal is to thoroughly cover the half of the plate with bacteria so that after incubation it will resemble the growth shown in Figure 44.1.

3 **Inoculate the Other Half of the Plate:** Being careful not to mix the cultures, repeat the inoculation process described in Step 2 to introduce the second test organism to the other half of the plate. Allow the agar to absorb the broth for 5 minutes before proceeding to the next step.

4 **Apply Disks to the Plate:** Alcohol-flame the forceps by placing them in the Bunsen burner flame long enough to ignite the alcohol, and then remove them. *Caution!* Do not hold the forceps in the flame and do not hold them near the alcohol jar. If the alcohol jar should ignite, smother the flame with the jar's lid. Once the alcohol has burned off, use the forceps to place the appropriate antibiotic disk in the center of the half containing the first organism. Gently tap the disk into place to ensure that it makes full contact with the agar surface, but do *not* push the disks into the agar. Return the forceps to the alcohol and repeat the process for the other half of the plate.

5 **Repeat for Every Plate:** Repeat the inoculation and disk placement process (Steps 2–4) for the other plates.

6 **Incubate Your Plates:** Incubate your inverted plates at 35±2°C for 24 to 48 hours.

7 **Clean up:** Dispose of the materials as your instructor directs, disinfect your work surface, and thoroughly wash your hands with soap and water before leaving the lab.

→ Next Lab Session

1 **Examine the Samples:** Remove the plates from the incubator (or refrigerator). Hold each plate over a black, nonreflective surface and examine the plate. The edge of a zone is where no growth is visible to the naked eye. Measure the diameter of each zone of inhibition in millimeters.

2 **Record Your Observations:** Use Tables 44.1, 44.2, and 44.3 to help you record your results in the data table at the end of this exercise.

3 **Clean up:** Dispose of the plates as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 44

DATA SHEET

Bacitracin, Novobiocin, and Optochin Susceptibility Tests

During the Lab

1 Record the zone of inhibition diameters (mm) in the data table provided. Then, use Tables 44.1, 44.2, and 44.3 to help you assess if the bacterium was susceptible (S) or resistant (R) in its response to the tested antimicrobics.

Antibacterial Agent	Organism	Zone Diameter (mm)	Susceptible (S) or Resistant (R)
Bacitracin			
Novobiocin			
Optochin			

Lab Related Questions

1 The main application of bacitracin, novobiocin, and optochin susceptibility testing procedures is to:

a. Identify if a bacterium is a pathogen.

b. Determine if the bacterium is Gram positive.

c. Determine which drugs would be best for treating an infection caused by the tested bacterial isolate.

d. Presumptively identify the tested bacterial isolate.

e. Determine if the tested bacterial isolate can break down red blood cells.

2 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ Bacitracin, novobiocin, and optochin susceptibility testing procedures typically call for TSA media.

_____ Bacitracin, novobiocin, and optochin susceptibility testing procedures are performed on mixed bacterial cultures.

_____ Bacteria that have a ZOI around a drug-infused disk are automatically considered susceptible to the tested antimicrobial drug.

_____ Bacteria that lack a ZOI around a drug-infused disk are automatically considered resistant to the tested antimicrobial drug.

3 Why is it important to grow a bacterial lawn in this experiment rather than isolated colonies on the plated media?

4 Select the true statement(s) (select all that apply).

a. Bacitracin is usually administered in an ingested pill format.

b. Bacitracin is associated with renal toxicity.

c. Bacitracin would be useful to treat an *S. aureus* wound infection.

d. Bacitracin is mainly used as a topical preparation.

e. Bacitracin tests allow for a presumptive identification of *Streptococcus pyogenes*.

5 Assume you know your tested bacterium is sensitive to bacitracin and α-hemolytic. Based only on this data, what can you rule out as the bacterium's identity? (Select all that apply.)

a. *Streptococcus pyogenes*

b. *Staphylococcus aureus*

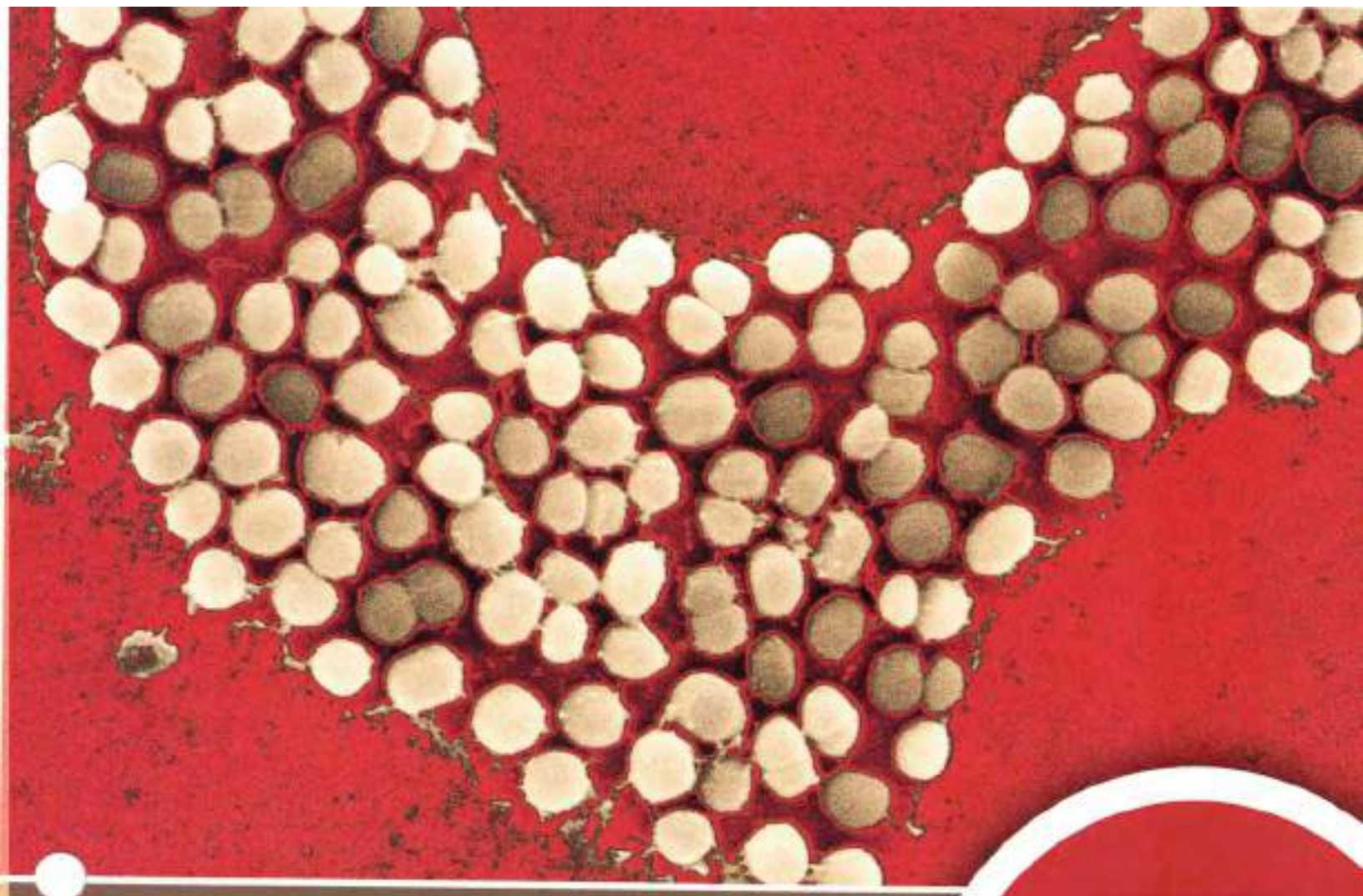
c. *Micrococcus luteus*

d. *Streptococcus pneumoniae*

Critical Thinking

1 Does the zone of inhibition's edge indicate the limit of the antibiotic's diffusion into the agar? Explain your reasoning.

2 What effect would using an older culture of *Streptococcus pyogenes* have on bacitracin susceptibility test results?



SECTION

8

Microbes Causing Trouble: Clinical Biofilms and Epidemics

In This Section

EXERCISE 45 Clinical Biofilms 409

EXERCISE 46 Epidemic Simulation 413

— Lab Notes —

EXERCISE 45

Clinical Biofilms

After doing this exercise, you should be able to:

1 Define the term *biofilm*.

2 State why biofilms represent a challenge in medicine.

3 Simulate the formation of a biofilm.

Background

A **biofilm** is a collection of one or more species of microorganisms and microbial products that have adhered to a surface. Biofilm development begins with formation of a **conditioning film** composed of biomolecules and particles from the environment on the surface. This results in the surface being more conducive to microbial adhesion. As **planktonic** (free-floating) microorganisms adhere to the surface, the biofilm grows.

The precise mechanism for cell adhesion varies depending on the amount of fluid movement and other factors; however, biofilm formation invariably depends upon the microbial community itself through secretion of an extracellular polysaccharide (**glycocalyx**). As more microbes attach, they contribute to the biofilm's chemistry, which in turn may further promote the adhesion of other microorganisms. As the biofilm matures, cells may detach from the film through erosion, abrasion, simple breakage, or physiological signals that promote planktonic cell release.

Biofilms occur in natural environments, but they also form in industrial and medical settings. Biofilm formation on indwelling medical devices (e.g., catheters) can lead to **health care-associated infections** (commonly called HAIs, or nosocomial infections), which are infections that develop while a patient is receiving care in a health-care facility. According to the U.S. Centers for Disease Control and Prevention, on any given day about one in every 31 hospital patients has one or more HAIs. Staphylococcal species are commonly associated with clinical biofilms, especially *Staphylococcus aureus* and *S. epidermidis*, which are normal residents of the skin microbiota and therefore easily transferred to medical devices.

The protective matrix and many layers of cells in a biofilm shelter the microbial residents from antimicrobial compounds and from the patient's immune defenses. Furthermore, microbes in biofilms seem to express antibiotic resistance factors at a greater level as compared to planktonic cells. This point combined with the physical sheltering from antimicrobics renders microbes in biofilms up to 1,000 times more resistant to antimicrobial drugs as compared to planktonic counterparts.[1] These features make biofilm-based infections especially challenging to eliminate. This point is even more alarming when we consider the estimate from the National Institutes of Health that biofilms account for 80% or more of microbial infections in the body.

[1] Gebreyohannes, G., A. Nyerere, C. Bii, and D. B. Sbhatu. "Challenges of intervention, treatment, and antibiotic resistance of biofilm-forming microorganisms," *Heliyon* 5 no. 8 (2019): e02192.

Clinical Applications

As previously mentioned, biofilms are a serious concern in medical settings, so understanding how they form is clinically important. *S. aureus* and *S. epidermidis* are notorious for forming biofilms on invasive medical devices, resulting in nosocomial infections.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Overnight broth cultures of *S. aureus* (BSL-2) and *S. epidermidis*
- ❑ Tubes of tryptic soy broth (TSB) enriched with 1% glucose (2)
- ❑ 50 mL phosphate buffered saline (PBS) working solution
- ❑ 50 mL 0.1% crystal violet
- ❑ Deionized water bottle
- ❑ Test-tube rack
- ❑ Receptacle for culture media disposal

Media, Stain, and Buffer Recipes

Tryptic Soy Broth (TSB) Supplemented with 1% Glucose

- Tryptic soy broth — 1.0 L
- Glucose — 10.0 g

0.1% Crystal Violet Stain

- Gram crystal violet (1%) — 10.0 mL
- dH_2O — 90.0 mL

10× Phosphate Buffered Saline (PBS)

- Na_2HPO_4, anhydrous, reagent grade — 12.36 g
- NaH_2PO_4-H_2O, reagent grade — 1.80 g
- NaCl, reagent grade — 85.00 g

Dissolve ingredients in distilled water to a final volume of 1 L

PBS Working Solution (0.01 M Phosphate, pH 7.6)

- 10× PBS stock solution — 100 mL
- dH_2O — 900 mL

PROCEDURE

You will grow a mixed culture of *S. aureus* and *S. epidermidis* for up to 1 week in a test tube, stain it, and observe for evidence of a biofilm. Wear a lab coat, gloves, and chemical eye protection when performing this procedure. *Caution!* Follow BSL-2 precautions throughout this lab.

1. **Obtain Two Tubes of TSB + 1% Glucose:** Label the first tube of media as "*S. aureus* with *S. epidermidis*." Label the second tube "negative control." Write your name and the date on both tubes.
2. **Inoculate the Media:** Heavily inoculate the first TSB + 1% glucose tube with both *S. aureus* and *S. epidermidis*. *Caution!* Use BSL-2 precautions when transferring *S. aureus*. Do not inoculate the second tube of media.
3. **Incubate Your Samples:** Incubate the tubes for 24–48 hours at 35 ± 2°C.
4. **Clean up:** Dispose of stock samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

8

→ **Next Lab Session**

1. **Obtain Your Samples and Decant the Liquid:** Place a disposal receptacle on a disinfectant-soaked towel. Then, carefully pour the broth media from each tube into the disposal receptacle. Avoid dripping the culture onto any surfaces. If you do spill or drip culture, clean it up with your lab's disinfectant and thoroughly wash your hands.
2. **Rinse the Tubes:** Gently rinse each tube with 1× PBS. Empty the buffer into the designated disposal receptacle. Repeat so that each tube is rinsed twice.
3. **Dry the Tubes:** Air-dry the tubes in an inverted position on a test-tube rack.
4. **Stain and Rinse the Tubes:** Stain both tubes with 0.1% crystal violet solution for 10 minutes at room temperature. To ensure full coverage of the tube, add more stain than the original volume of broth in the tubes. After 10 minutes, pour the dye into the designated receptacle. Then gently fill each tube with dH_2O and immediately pour the water into the designated receptacle. This rinse will remove unbound crystal violet. Again, air-dry the tubes in an inverted position.
5. **Examine Your Samples:** Compare your results with Figure 45.1. You are looking for a purple film adhering to the inside of the experimental tube. Ignore any dark ring at the top of the tube.
6. **Record Your Result:** Record your results in the data sheet at the end of this exercise.
7. **Clean up:** Dispose of the samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

FIGURE **45.1** **Staphylococcal Biofilm** • The faint purple is the biofilm stained with dilute crystal violet. The tube on the right is an uninoculated control.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 45

DATA SHEET

Clinical Biofilms

During the Lab

1 Record your results for the biofilm test in the following table.

Tube	Observations
Control	
Staphylococcus mixture	

Lab Related Questions

1 A biofilm is best described as:

a. A community of microbes in a solution.
b. A community of microbes that causes disease.
c. A community of microbes attached to an indwelling medical device.
d. A community of microbes adhered to a surface.
e. A community of antibiotic-resistant microbes.

2 Free-floating bacteria are called:

a. Plantae.
b. Plankton.
c. Pluralistic.
d. Planktonic.
e. Planktic.

3 List two reasons why microbes in biofilms are difficult to eliminate.

_____

_____

_____

4 Assume you saw biofilm formation in the control tube. What could account for such an observation? (Select all that apply.)

a. Your TSB-glucose media was contaminated.
b. The dye stuck to the glucose in the media.
c. The crystal violet dye solution was contaminated.
d. You left the tubes in the incubator too long.
e. You used non-sterile test tubes.

5 A polysaccharide-enriched extracellular structure called the _____ assists in biofilm formation.

Critical Thinking

1 Assume you forgot to add glucose to the TSB media. What results would you anticipate and why?

2 Suppose you forgot to add *S. epidermidis* to the media and instead double inoculated with *S. aureus*. What result would you expect? Explain your reasoning.

EXERCISE 46

Epidemic Simulation

After doing this exercise, you should be able to:

1. Define the term *epidemiology* as well as other basic terms such as *fomites*, *portal of entry*, and *index case*.
2. Describe the difference between direct and indirect transmission and provide examples of each.
3. Calculate the incidence and period prevalence of a simulated epidemic.
4. Determine the index case of a simulated epidemic.
5. Perform a simulated epidemic and interpret the test results.

Background

Epidemiology is the study of the causes, occurrence, transmission, distribution, and prevention of infectious and noninfectious diseases in populations. While there is no single approach to finding answers to these epidemiological questions, identification of the pathogen frequently happens first followed by an exploration of the pathogen's transmission mode.

Pathogens are transmitted to new hosts through direct or indirect mechanisms. **Direct contact transmission** occurs when a host is infected during a direct physical interaction with an infected host or an environmental source (e.g., person is bitten by an infected animal; sexual contact with an infected individual; vertical transmission from an infected mother to her fetus; bacteria in soil directly entering a wound). In contrast, **indirect contact transmission** does not require a physical encounter with an infected host. Instead, the infection may spread through the air (e.g., respiratory aerosols emitted by the coughs or sneezes of an infected person); via **fomites** (inanimate objects) like contaminated surfaces or dirty needles; through food and water (e.g., norovirus may get into food and spread without ever directly encountering an infected person); and via vectors (e.g., cockroaches, biting insects, etc.). The route the pathogen uses to gain access to the host's body and establish infection is referred to as a **portal of entry** (e.g., through the GI tract or respiratory tract). Some pathogens have more than one portal of entry.

When a disease is transmitted from a specific source to a population (e.g., a cooling system of a building spreading a pathogen to many inside the building), the resulting outbreak is called a **common source epidemic. Propagated transmission** is where the pathogen is transmitted from person to person. The **index case** (or patient 0) is the first documented disease case in an outbreak.

In this exercise, you will determine the index case of a simulated epidemic. You will also gather data and calculate **incidence** and **prevalence rates** for the "disease" spreading through your class.

Incidence rate is essentially a measure of the frequency of *new* disease cases in a population within a specified time period. We use the following equation to calculate incidence rate:

$$\text{Incidence rate} = \frac{\text{Number of new disease cases in a specified time period}}{\text{Number of people at risk for the disease at the midpoint of the time period}} \times 10^n$$

Because population size constantly changes, it is conventional to use the population size at the midpoint of the study period. Also, the usually small decimal fraction obtained for the rate is multiplied by some power of 10 (10^n) to get its value up to a number bigger than one. That is, an incidence rate of 0.00003 is multiplied by 100,000 (or 10^5) so it can be reported as three cases per 100,000 people per time period, rather than 0.00003 cases per person per time period.

You also will be calculating one form of prevalence rate called **period prevalence.** This measure helps us understand the burden of disease in a population during a specified period. Period prevalence is calculated as follows:

$$\text{Period Prevalence Rate} = \frac{\text{Number of existing cases in a specified time period}}{\text{Number of people at risk for the disease at the midpoint of the time period}} \times 10^n$$

As with incidence rate, the calculated prevalence is multiplied by some power of 10 (10^n) to bring the number up to one or more. Alternatively, if the average duration (D) of a disease and the incidence rate (I) are known, then prevalence (P) can be estimated as follows:

$$P = I \times D$$

Clinical Applications

Epidemiologists have the task of identifying the source of a disease and discovering the mode of its transmission. Further, epidemiologists characterize diseases quantitatively, using measures such as incidence and prevalence. Incidence rate indicates the probability an individual will contract the disease in the stated time, whereas prevalence is a useful indicator of the disease's presence in a population. Based on these and other information, epidemiologists can make recommendations for behaviors that can reduce disease transmission (e.g., boiling water before drinking it, wearing a mask while in public, etc.). Epidemiological data can also help medical scientists as they develop drugs, vaccines, and other therapeutics for specific diseases.

Procedure Modification

We selected *S. marcescens* as the transmissible agent in this lab because it is a BSL-1 bacterium with a distinctive color that is easy to detect upon culturing. ***Note:*** This bacterium is known to be an opportunistic infectious agent and proper precautions must be observed. To **avoid possible health risks, instructors can use Glo Germ powder or Glo Germ gel rather than bacteria.** In such a case, rather than noting bacterial growth as the measure of infection, UV light is used to detect Glo Germ on gloves after all handshakes in the simulation procedure have occurred. The Glo Germ-based procedure requires only one lab session whereas the bacteria-based procedure requires two sessions.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Each student will need a numbered petri plate containing one hard candy. Only one of the plates should contain a contaminated candy (a piece of candy swabbed with a culture of *Serratia marcescens*[1]); the other plates will contain a non-contaminated candy (candy swabbed with sterile saline). Number the petri plates according to the number of students in the class. Do not leave gaps in the numbering sequence.
- ❑ Tube of sterile saline (1 per student)
- ❑ Nutrient agar (NA) or tryptic soy agar (TSA) plate (1 per student)
- ❑ Sterile cotton-tipped swabs (2 per student)
- ❑ Marking pen
- ❑ Containers for swab and glove disposal should be located around the room so all students have easy access.

[1] Your instructor may substitute Glo Germ powder or gel for the "contaminant" on the candy. If this is the case, then sterile swabs and growth media are not necessary. Instead, after the contact transmission simulation, students use UV light to check if their gloved nondominant hand is contaminated with the glowing substance.

MATERIALS FOR GLO GERM ADAPTATION OF THE PROCEDURE

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Glo Germ powder or gel (sold by numerous vendors, including Carolina Biological and Fisher Scientific)
- ❑ Each student will need a numbered petri plate containing one hard candy. Only one of the plates should contain a contaminated candy (a piece of candy dipped in Glo Germ gel or powder) the other plates will contain a non-contaminated candy (candy swabbed with sterile saline) Number the petri plates according to the number of students in the class. Do not leave gaps in the numbering sequence.
- ❑ UV light to check for Glo Germ on gloved nondominant hand after the simulation

PROCEDURE

For your safety and for the safety of those around you, carefully follow the instructions in this lab. For enhanced safety, the instructor should orchestrate the entire lab so that steps are precisely performed, and students do each step at the same time. Before you begin, look at Figure 46.1 to see how to separate gloves safely. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

Bacteria-Based Simulation Procedure

1. **Obtain the Materials:** Each student should obtain a petri plate containing candy, two sterile cotton-tipped swabs, a tube of sterile saline, and a nutrient agar or TSA plate. Circle the number of your candy plate in the "case" column of the data sheet at the end of this lab exercise. Once you have noted your "case number" put away all your papers and books until the lab is finished.
2. **Label the Media:** Use a marking pen to label the bottom of your petri plate with your name and the number of your candy plate. Then draw a line to divide the plate in half. Label the halves as "Side 1" and "Side 2," respectively.
3. **Open the Applicators:** Open a package of sterile, cotton-tipped swabs so they can be easily removed. You'll only need two swabs for this lab.
4. **Pre-Transmission Sampling of the Palm of Your Nondominant Gloved Hand:** After putting on your gloves, use your dominant hand to remove one

cotton-tipped swab, moisten it in the sterile saline, and sample the palm of your other gloved hand. Do not touch anything else with the palm of the glove. When finished, zigzag inoculate Side 1 of the plate with the cotton-tipped swab (see Fig. 4.6, p. 72). Discard the swab in an appropriate container. This side will serve as your pre-exposure control.

5 **Pick up the Candy:** Pick up the candy with your gloved nondominant hand. Roll the candy around in your hand until a good amount of it is transferred to the glove. Then, drop the candy back into the petri plate and close the lid with your dominant hand. **Touch nothing with your "contaminated" hand!** Just sit with your elbow on the table and your hand in the air. Be mindful that YOU might be the one with *S. marcescens* on your hand.

6 **Transmission Simulation Round 1:** The plate number identifies each student as a case number. Using "contaminated" hands only, Case #1 rubs hands with Case #2, making sure to transfer anything that may be on the palm of the glove to the palm of the other student's glove. Both students must hold on to the cuff of their glove throughout this step so it doesn't come off. After a few seconds of rubbing, gently slide gloved hands apart, as shown in Figure 46.1. **Do not "snap" the gloves as they separate because this will produce aerosols.** Case #1 should go back to their desk and sit with the elbow of their "contaminated" hand on the table so that their hand is in the air and not making contact with anything.

7 **Subsequent Transmission Simulations:** Next, Case #2 rubs gloved hands with Case #3, and #3 with #4, and so on until all students have contacted "contaminated" hands. Continue to be careful not to snap the gloves when you separate. Hold on to the cuff when transferring and separating to prevent the glove from coming off your hand.

8 **Ending the Transmission Simulation:** When all students have rubbed hands, the last (highest numbered) case should rub hands with Case #1.

9 **Post-Transmission Sampling of the Palm of your Gloved Nondominant Hand:** With a second sterile cotton-tipped swab, sample the palm of your "contaminated" hand as before and inoculate Side 2 of the plate. Be sure to sample the area of the glove that contacted other students' gloves. Dispose of the cotton swab in the nearest appropriate autoclave container. Do not walk across the room with the swab.

10 **Remove Your Gloves:** Remove your gloves by inserting the thumb of your dominant hand under the cuff of the "contaminated" glove and rolling it off your hand, inverting it in the process (see Fig. S.4, p. 6). Remove the other glove using the same rolling method. Dispose of the gloves properly.

11 **Wash Your Hands:** Wash your hands thoroughly (preferably with antiseptic soap, if available).

12 **Incubate Your Sample:** Tape the lid on your inoculated plate. Invert the inoculated plate and incubate it at 25°C for 24 to 48 hours.

13 **Clean up:** Discard all materials in appropriate containers. Be sure to tape the lid on your candy plate before you dispose of it. Wipe down your work area with disinfectant.

14 **Wash Your Hands *Again*:** After cleaning up, thoroughly wash your hands again.

FIGURE **46.1** **Sliding the Gloves Apart** • After rubbing palms, students must separate by gently sliding their gloved hands apart (as shown in A and B). Hold on to the glove's cuff to prevent it from coming off. These safety precautions must be followed and are designed to prevent the gloves from snapping apart (they will be sticky) and producing aerosols.

→ Next Lab Session

1. **Obtain Your Samples:** Remove the plates from the incubator and examine them for characteristic reddish growth of *S. marcescens*.
2. **Examine Your Samples:** Examine Side 1 of your plate for the characteristic red-orange growth of *S. marcescens*. Notify your instructor if you see *S. marcescens* growth. If your plate shows *S. marcescens* growth on Side 2, enter a "+" next to your case number on the data sheet at the end of this exercise. If there is no evidence of *S. marcescens* growth, enter a "–" instead.
3. **Share Your Result:** All class members will need to report if they had *S. marcescens* growth on Side 2 of their plate. Your instructor may do a roll call, have you post results on the board, or use some other mechanism for dispensing this information. Enter class results on your data sheet at the end of this exercise, and follow the instructions for establishing the index case, incidence rate, and period prevalence.
4. **Clean up:** Dispose of the samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

Glo Germ-Based Simulation Procedure

1. **Obtain the Materials:** Each student should obtain a petri plate containing a candy. Circle the number of your candy plate in the "case" column of the data sheet at the end of this lab exercise. Once you have noted your "case number," put away all your papers and books until the lab is finished.
2. **Pre-Transmission Observation of Nondominant Gloved Hand:** After putting on your gloves, observe your nondominant gloved hand under the UV light. If you observe any glowing, wash your gloved hand with plain water. After air-drying the gloved hand, reobserve it under the UV light to ensure your hand is "glow free" at the start of the simulation. Once you have established that your nondominant hand is free of any glowing substance(s), do not touch anything else with the palm of the glove.
3. **Pick up the Candy:** Pick up the candy with your gloved nondominant hand. Roll the candy around in your hand until a good amount of it is transferred to the glove. Then, drop the candy back into the petri plate and close the lid with your dominant hand. **Touch nothing with your "contaminated" hand!** Just sit with your elbow on the table and your hand in the air. Be mindful that YOU might be the one with the Glow Germ on your hand.
4. **Transmission Simulation Round 1:** The plate number identifies each student as a case number. Using "contaminated" hands only, Case #1 rubs hands with Case #2, making sure to transfer anything that may be on the palm of the glove to the palm of the other student's glove. Both students must hold on to the cuff of their glove throughout this step so it doesn't come off. After a few seconds of rubbing, gently slide gloved hands apart, as shown in Figure 46.1. **Do not "snap" the gloves as they separate because this will produce aerosols.** Case #1 should go back to their desk and sit with the elbow of their "contaminated" hand on the table so that their hand is in the air and not making contact with anything.
5. **Subsequent Transmission Simulations:** Next, Case #2 rubs gloved hands with Case #3, and #3 with #4, and so on until all students have contacted "contaminated" hands. Continue to be careful not to snap the gloves when you separate. Hold on to the cuff when transferring and separating to prevent the glove from coming off your hand.
6. **Ending the Transmission Simulation:** When all students have rubbed hands, the last (highest numbered) case should rub hands with Case #1.
7. **Observation of Your Gloved Nondominant Hand:** Place your gloved nondominant hand under a UV light to check for Glo Germ. If you observe glowing particles on the glove, enter a "+" next to your case number on the data sheet at the end of this exercise. If there is no evidence of *glowing*, enter a "–" instead.
8. **Remove Your Gloves:** Remove your gloves by inserting the thumb of your dominant hand under the cuff of the "contaminated" glove and rolling it off your hand, inverting it in the process (see Fig. S.4, p. 6). Remove the other glove using the same rolling method. Dispose of the gloves properly.
9. **Clean up:** Discard all materials in appropriate containers and thoroughly wash your hands.
10. **Share Your Result:** After the simulation is completed, all class members will need to report if they had Glo Germ on their nondominant hand. Your instructor may do a roll call, have you post results on the board, or use some other mechanism for dispensing this information. Enter class results on your data sheet at the end of this exercise, and follow the instructions for establishing the index case, incidence rate, and period prevalence.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 46

DATA SHEET

Epidemic Simulation

During the Lab

1 Before You Begin: Circle your "case number" in the following table. Your case number is written on the petri plate you were given that contains the candy.

Case	Week	Disease (+/–)	Case	Week	Disease (+/–)	Case	Week	Disease (+/–)	Case	Week	Disease (+/–)
1	1		11	3		21	4		31	7	
2	1		12	3		22	4		32	7	
3	1		13	3		23	5		33	7	
4	1		14	3		24	5		34	7	
5	2		15	3		25	5		35	8	
6	2		16	4		26	5		36	8	
7	2		17	4		27	5		37	8	
8	2		18	4		28	6		38	9	
9	2		19	4		29	6		39	9	
10	3		20	4		30	6		40	9	

2 If you used the Glow Germ procedure: Note glowing on the gloved nondominant hand with a "+" as an indication of infection. If there is no glowing, consider the person to be healthy and record a "–" in the appropriate box.

3 If you did NOT use the Glo Germ procedure, analyze the data as follows:

a. **Observe Side 1 of your plate:** After the plates have been incubated, compile data for your class population (each case is the student's candy number in the exercise). The organism spread during contact between student pairs was *Serratia marcescens*, which produces reddish-orange colonies. Check Side 1 of your plate for evidence of *S. marcescens* growth. Notify your instructor if you see any.

b. **Observe Side 2 of your plate:** You will likely see growth on both sides of the plate, but red colonies are the only sign of "disease." If there is reddish-orange growth on Side 2 of the plate, consider the person to have the "disease" and record a "+" in the appropriate box. If there is no red growth on the plate or there is no difference between the two sides, consider the person to be healthy and record a "–" in the appropriate box.

4 After reviewing all the class data, deduce which case is the index case:

Case# _____ was the index case.

5 **Use the class data to calculate the incidence and period prevalence rates of your class population:** For this purpose, assume that the cases have been identified over a 9-week period (or a shorter period, depending on how many students participated in the simulation). Also assume that the duration of the disease is 7 days, so new cases in 1 week are still diseased in the next week but are healthy by the following week.

Record the population size: _____

Use the table provided to record incidence and point prevalence rates for each week. The incidence and period prevalence rates should be reported as "cases per 10^n per week."

Week	Incidence Rate	Period Prevalence Rate
1		
2		
3		
4		
5		
6		
7		
8		
9		

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 46
DATA SHEET
(continued)

Lab Related Questions

1 Choose the true statement(s) (select all that apply).

a. Epidemiologists perform work that reveals the source of a disease.
b. Epidemiology includes the study of noninfectious disease occurrence in populations.
c. Epidemiologists develop vaccines for diseases.
d. Epidemiology includes the study of infectious disease occurrence in populations.
e. Epidemiological data includes information on how diseases are transmitted in populations.

2 Which of the following disease features would contribute to a higher incidence rate? (Select all that apply.)

a. A noninfectious agent was the cause of disease.
b. An infectious causative agent was the cause of disease.
c. The disease is mainly transmitted by indirect contact.
d. The disease is mainly transmitted by direct contact.
e. The disease is transmitted the best before obvious signs and symptoms manifest in an infected person.

3 Assume you conducted the simulation as described in this exercise, but with only 6 total students. The data reveal that only Students #2 and #3 remained uninfected. In such a scenario, who was the index case?

a. Student 1
b. Student 3
c. Student 4
d. Student 5
e. Student 6

4 The index case is best described as:

a. The first patient to die of a particular disease in a population.
b. The first documented disease case in an outbreak.
c. The first patient ever diagnosed with a given disease.
d. The first patient to receive a vaccine against a given disease.
e. The first patient to exhibit symptoms of a specific disease in a population.

5 If you know the period prevalence rate and the duration of a disease then you know/can determine (select all that apply):

a. Incidence rate.
b. Mode of transmission.
c. The number of people at risk for the disease at the midpoint of the time period.
d. The index case.
e. The disease source.

6 During the COVID-19 pandemic, a simple calculation called the *percent positive* was a useful figure for understanding disease burden in a population. In most cases, the recommendation was that health departments work to maintain a percent positive of less than 5%. The percent positive is calculated as follows:

$$\text{Percent Positive} = \frac{\text{Number of people who had a positive test result in a specified time period}}{\text{Total number of people tested in the time period}} \times 100$$

Based on this information, which scenario(s) would increase the percent positive measure? (Select all that apply.)

a. Decreased disease cases in a population and increased testing in that same population.
b. Decreased disease cases in a population and decreased testing in that same population.
c. Increased disease cases in a population and increased testing in that same population.
d. Increased disease cases in a population and decreased testing in that same population.

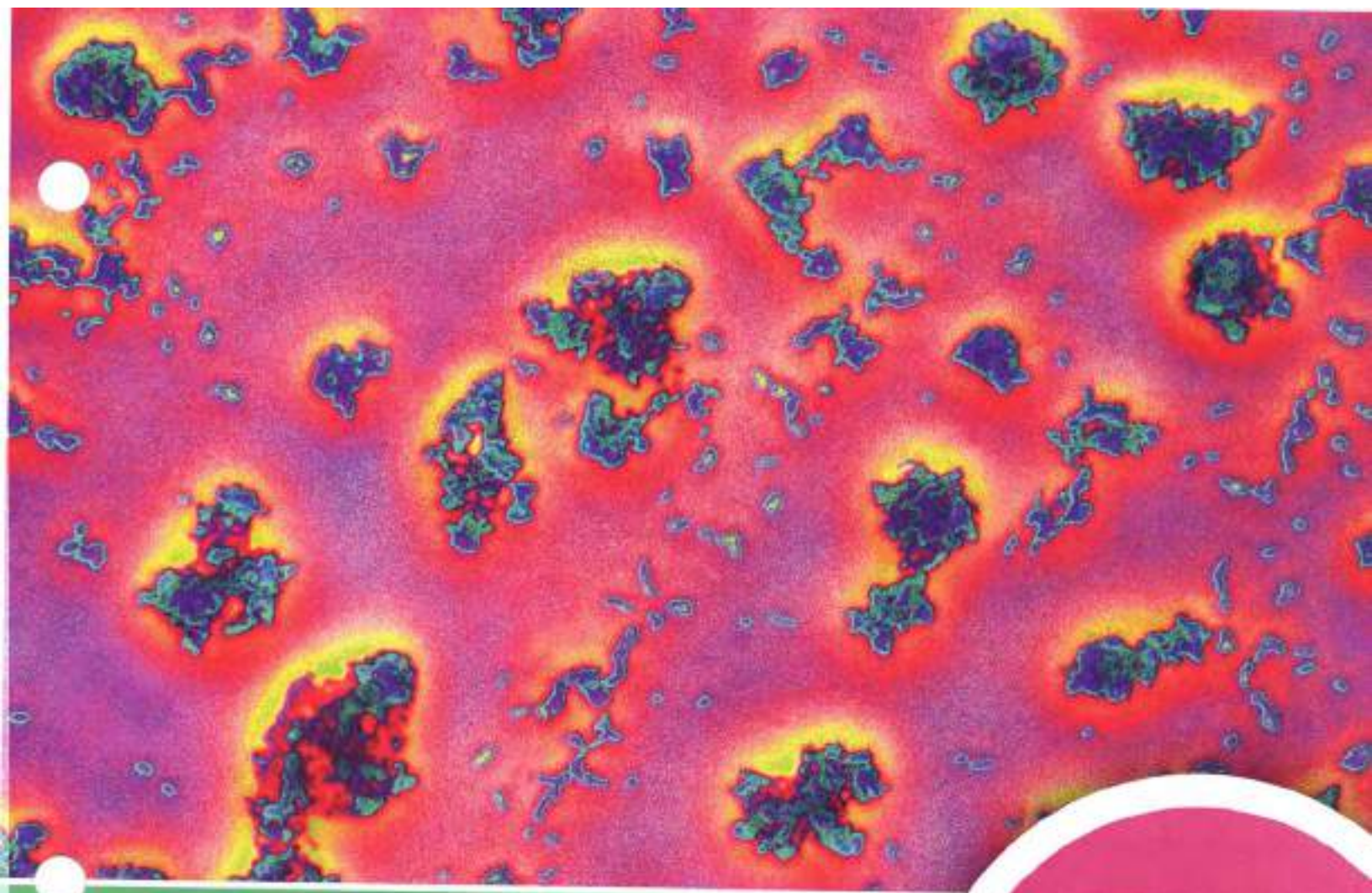
7 Assume 10 students participated in the simulation described in this lab exercise and the index case was Student #10. In such a scenario, who among the following should show evidence of being "infected" in the simulation? (Select all that apply.)

a. Student 1
b. Student 2
c. Student 8
d. Student 9
e. Student 10

Critical Thinking

1 We made numerous assumptions about the nature of the "disease" in this simulation. List three assumptions that were inherent to the simulation.

2 If a disease's average duration remains constant, then how would an effective preventive measure that reduces disease incidence likely affect disease prevalence? Explain your reasoning.



SECTION

9

Identifying Unknowns

In This Section

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— Lab Notes —

EXERCISE 47

Case-Based Unknown (Dry Lab)

After doing this exercise, you should be able to:

1. Discuss three misconceptions that you should avoid if using a dichotomous key identification strategy.
2. Explain why a battery of biochemical tests is normally inoculated for identifying an unknown bacterium.
3. State what the API test system is and how it generally works.
4. Apply your knowledge of biochemical tests to determine the identity of a theoretical unknown.

Background

The underlying goal of all clinical microbiology laboratory efforts is to provide accurate information as quickly as possible, delays and inaccurate data cost lives. Data accuracy is in no small part affected by the sample collection process at the point of care; it makes no difference how skilled the microbiology team is if the health-care provider fails to practice proper aseptic techniques and sample collection protocols. During the laboratory part of your microbiology training, you have been practicing aseptic techniques that are essential to saving lives; you'll apply them in patient care as well if you are required to collect a sample for microbiological analysis.

Most of the methods you have learned about in this laboratory course are microscopy- and culture-based. While such methods remain valid assessment tools, they are often accompanied by a wide variety of molecular-based tools (e.g., genetic analysis, serological studies, etc.).

Ultimately, clinical suspicion drives what tests the clinical microbiologist will perform. That is, the clinician collects specific patient samples (e.g., blood, urine, etc.) based on the nature of the patient's signs and symptoms. For example, a urine sample is collected when a urinary tract infection (UTI) is suspected. A throat swab is performed when strep throat (streptococcal pharyngitis) needs to be ruled out. A sample of cerebrospinal fluid is often collected if the patient is experiencing signs and symptoms of meningitis. Because clinical suspicion is really the driving force for clinical microbiological analysis, it's worthwhile for you to perform an "unknown" analysis with some infusion of that context. In this lab, you'll follow a patient from signs and symptoms to definitive diagnosis using microscopy and biochemical test data to inform clinical decisions.

Dichotomous Key Strategies: Avoiding Misconceptions

Dichotomous keys, like that shown in Figure 47.1, are a common approach to teaching students about identifying a bacterial unknown.[1] These branching-style maps are useful because they provide a visual representation of how one species differs from another. In that spirit, some of you may be asked to develop a dichotomous key to show that you understand how certain bacteria differ from one another. While dichotomous keys have their place in bacterial identification, they have the potential to perpetuate several misconceptions about the bacterial identification process:

1. Students may see such keys and conclude that one biochemical test is performed at a time until the bacterium's identity is determined;
2. Dichotomous keys imply a rank-and-file order/hierarchy for performing biochemical tests (always start with test A, then do B, followed by C, etc.); and
3. Students may perceive that tests earlier in the key are more accurate/reliable than those situated lower in the key.

The reality is that once a pure culture is obtained, a battery of biochemical tests is normally inoculated all at once and interpreted as soon as possible. There is not a stepwise, test-by-test inoculation. There are good reasons for this:

1. If clinical lab workers embraced a stepwise approach (inoculated one or two biochemical tests per day based on the data of a previous test), it may be more economical in terms of resources used, but it could *easily* triple the time required to determine the bacterium's identity.

[1] In addition to the dichotomous key in this exercise, there is a collection of dichotomous keys provided in the appendix of this book. Your instructor may have you use them for other lab exercises.

2. The types of tests performed hinge largely on clinical suspicion. For example, the clinical lab team will not analyze a urine sample with the intention of differentiating pathogens that cause UTIs from those that cause strep throat. That is, not all bacteria are reasonable suspects, the analysis is almost always narrowed based on the clinical presentation. In such cases, there is usually an empiric battery of tests—tests that experience has repeatedly shown are likely to lead to the correct identification of the culprit. Of course, if those empiric tests are inconclusive (which *can* happen), then further testing is necessary. That said, such a dead end would be detected in 24–48 hours by the battery approach versus a week of "if/then" testing that occurs in stages; time saved is lives saved.
3. No test is 100% accurate 100% of the time. Based on your experiences in the microbiology lab, this point may seem obvious; after all, you've learned there are always sources of error that can range from media formulations to strain variations to everything in between. Based on this fact alone, it doesn't make sense to hinge your testing workflow on a one-test-at-a-time strategy. What if the first test you performed yielded inaccurate results? You would probably waste a lot of time before figuring out the error, if you figured that out at all. Thus, you are *far* more likely to correctly identify your unknown if you perform a *collection* of tests and see where the closest match lies based on the various pieces of data.
4. Even if a particular bacterial species is noted as being positive for a particular test, that doesn't mean all isolates of the species are positive; the room for variation is often 10%. As many as 10% of the strains of a species listed as positive or negative on a test give the opposite result. That is, 90% of strains (genetic variants of the same species) may have a positive test result for a specific biochemical test while 10% of isolates may generate a negative test result. Indeed, perfect alignment is exceedingly rare. So, embarking on a

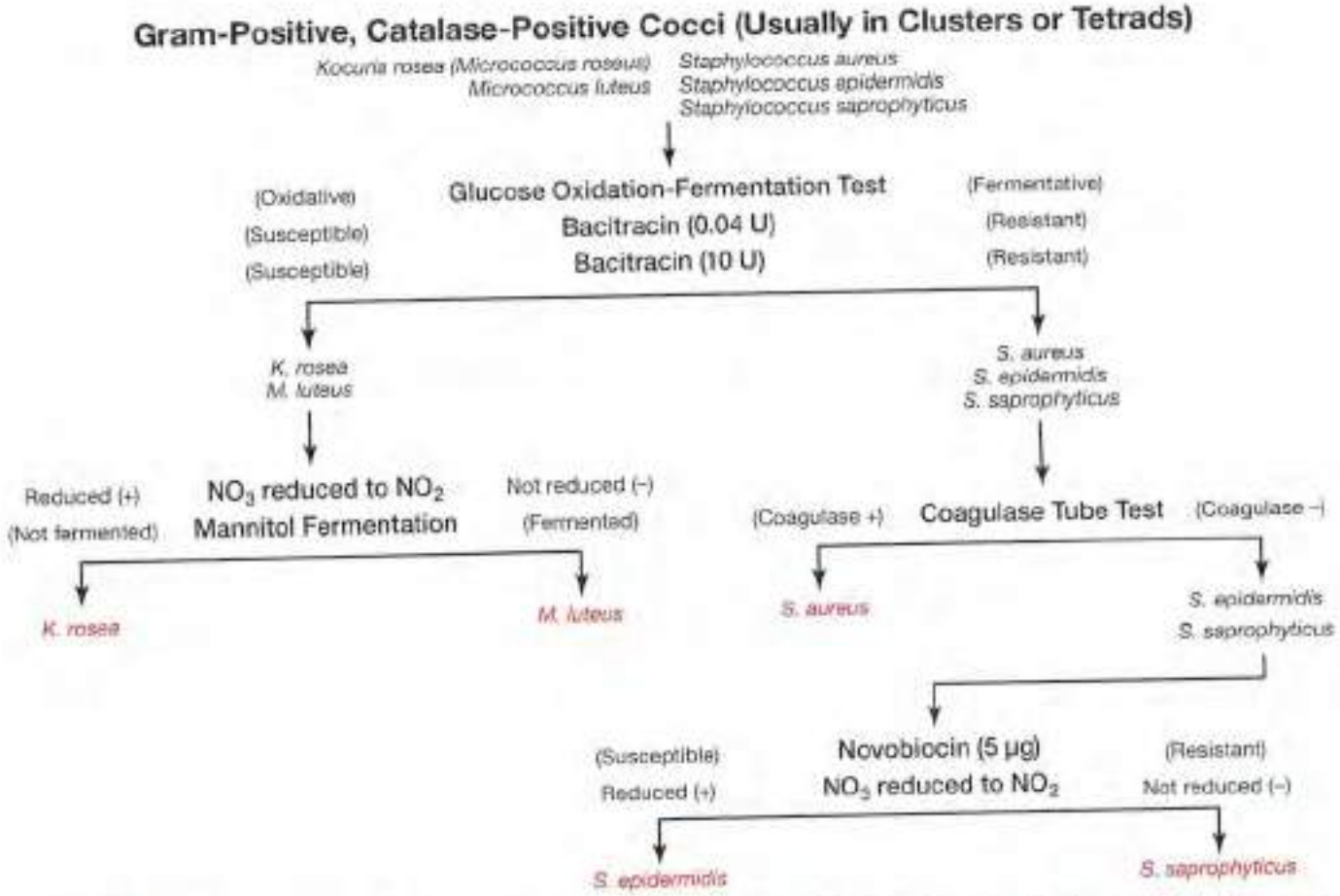


FIGURE 47.1 Dichotomous Key Flowchart for Gram-Positive, Catalase-Positive Cocci (Usually in Tetrads or Clusters) ■ A dichotomous key approach can be useful for bacterial identification. For example, this dichotomous key could be useful for discovering the identity of a pure culture of Gram-positive, catalase-positive, cocci arranged in clusters (or tetrads).

one-test-at-a-time strategy increases the risk of inaccurately identifying the isolate. It's much safer to consider all the data together rather than taking a piecemeal approach.

Based on these four points, when analyzing biochemical test results many clinical labs lean toward a "closest fit" approach rather than a traditional dichotomous key approach. In the closest fit approach, the data from a battery of biochemical tests are entered into a computer program, which then compares the reported data to one or more databases to determine the statistically most likely candidate (or candidates). One of the more widely used examples of this technology is the API system (Analytical Profile Index system manufactured by bioMérieux) (Fig. 47.2).

To more closely simulate the clinical lab bacterial identification strategy, this lab exercise will not use a dichotomous key strategy. You will instead analyze a clinical case, decide which battery of tests are most appropriate to isolate and identify the potential pathogen, and proceed from there with your analysis to determine the specimen's identity.

Clinical Applications

This lab provides a way for you to walk through the clinical specimen identification process. The process of identifying a pathogen in a sample typically begins with a health-care provider making the decision to collect a sample for microbiological analysis. That decision is usually made based on the signs and symptoms the patient is experiencing. Once the sample is collected, it is conveyed to a clinical microbiology lab. There the specimen is typically inoculated onto various media for isolation. Once an isolated candidate is grown, it is usually subjected to staining and microscopy to ascertain features like Gram property, cell shape, and cellular arrangement. Then, if the sample requires further analysis to make a definitive identification, it may be subjected to further biochemical testing.

MATERIALS

Because this is a "dry lab" (meaning the exercise doesn't require reagents or other lab materials), it can be an excellent way to gain a better grasp of the process before you are required to perform a bacterial identification lab with a real sample. Dry labs are also a great way to practice your problem-solving skills before you are called to use those skills in higher-stake settings with possibly dangerous biological and chemical substances.

api 50 CH
20 21 22 23 24 25 26 27 28 29
api 20 E
ONPG ADH LDC ODC CIT H2S URE TDA IND VP GEL GLU MAN INO SOR RHA SAC MEL AMY ARA
api 20 STREP
VP HIP ESC PYRA αGAL βGUR βGAL PAL LAP ADH RIB ARA MAN SOR LAC TRE INU RAF AMD GLYG

47.2 API Test Strips • Although only three examples of API test strips are shown, many other API test strips are available; most deliver test results in fewer than 24 hours. Before reading the test results, the microbiologist processing the samples would add specific reagents to certain cupules on the strip. If you look closely, you may notice that under each cupule there is an abbreviation that represents the test name. You may even recognize some of them. For example, CIT is the citrate test and GLU stands for the glucose fermentation test.

PROCEDURE

Read the case sections and perform the analyses as instructed.

At the Patient's Bedside

A 68-year-old man is brought to the emergency department (ED) of the hospital where you work. He reports a severe stabbing pain in his lower back, nausea without vomiting, and blood in his urine. CT scan imaging revealed two kidney stones (renal calculi) in the left kidney, as well as one stone lodged in the urethra and another stuck in the distal portion of the left ureter. He is running a fever and has underlying health conditions such as diabetes and heart disease. The ED doctor orders urine and blood samples to be collected for microbiological analysis as well as for other tests. Once the samples are collected, the doctor starts the patient on a broad spectrum intravenous antibacterial drug and admits him for inpatient hospital care.

In the Clinical Microbiology Lab

Day_0: Same Day as Sample Collection

You are responsible for analyzing the patient's urine sample and detecting the pathogen of interest. You start with a Gram stain of the centrifuged urine sample. You observe the following via light microscopy (Fig. 47.3):

FIGURE **47.3** Gram Stain of Centrifuged Urine Sample •

1. Gram property = _____
2. Cell shape = _____
3. Cellular arrangement = _____
4. Assuming oil immersion microscopy with a 10× ocular and 100× objective, what would the final magnification be for the image? _____
5. Based on the Gram-stain and morphological information, you can make an informed decision about your next steps. For each of the following, state if you would or would not perform the procedure and explain the reasoning behind your decision. Remember, performing unnecessary tests takes time and costs your lab money, so you would only perform tests that would help you further narrow down the bacterium's identity.

   a Inoculate the sample onto mannitol salt agar (MSA).

   b Test the bacteria for coagulase.

   c Test the bacteria for oxidase.

   d Perform an acid-fast stain.

   e Inoculate the sample onto eosin methylene blue agar (EMB).

At the Patient's Bedside

The infectious disease specialist familiarizes themselves with their newly admitted patient's case. In the meantime, the assigned urologist also visits the patient. They consult on the care plan for their shared patient. Their plan primarily centers on removing the lodged kidney stone within the next 12 hours while simultaneously managing the infection that has developed. The infectious disease doctor states that the Gram-stain data suggests a narrower-spectrum drug might be a more desirable option as compared to the broad-spectrum drug started in the ED.

However, until they have more data on the identity of the bacterium causing the infection and its antimicrobial drug susceptibility profile, they will keep the patient on the broad-spectrum antibacterial drug initiated in the ED.

6 Look up information on narrow-spectrum versus broad-spectrum drugs. Explain why a narrow-spectrum drug could be preferable to a broad-spectrum drug?

7 Based on this information, how would you likely describe the coloration of the growth on MacConkey agar? Explain the reasoning behind your response. (*Note:* To answer this question, assume you are only describing the color of a single colony of the suspected pathogen. Do not concern yourself with colony morphology.)

In the Clinical Microbiology Lab

Day$_1$: Approximately 24 hours after Sample Collection

Upon arriving at the lab today, you check your various cultures from yesterday. You observe that you have successfully isolated a bacterium that could be the pathogen responsible for the patient's urinary tract infection. The suspected bacterium appears to ferment lactose when grown on MacConkey agar.

Today your work will focus on fine tuning the bacterium's identity and performing drug susceptibility testing. Provided all goes well, you should know the bacterium's identity and drug susceptibility data by tomorrow, at which point you can send the microbiology report to the attending health-care team.

The lab where you work has an API system. This helps to reduce the time for identification because the tests are miniaturized and require shorter incubation periods. Some of the API test strip panels can return biochemical test results in as few as 4 hours. To help you decide which API test strip you should use, you refer to the following chart (Fig. 47.4):

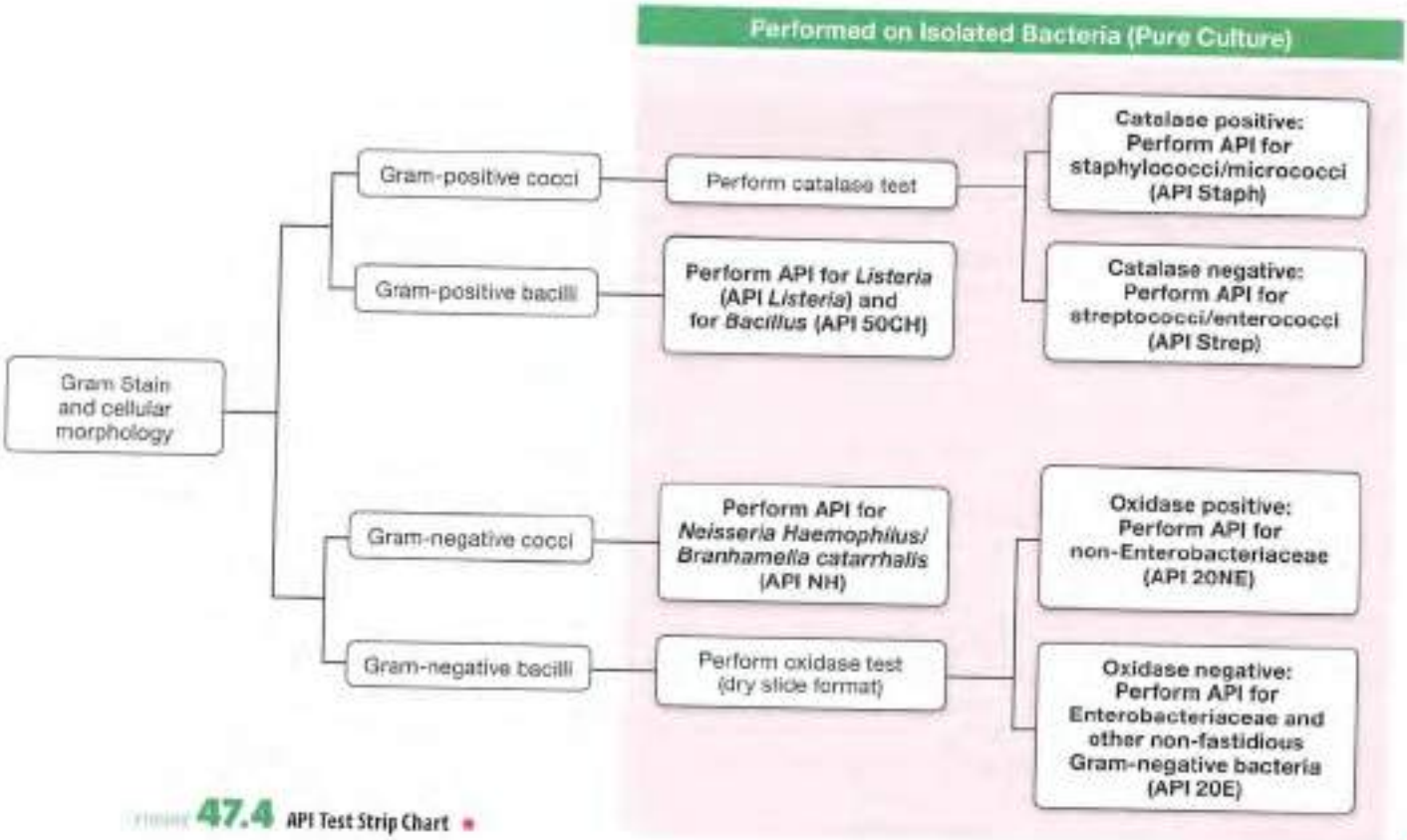

Performed on Isolated Bacteria (Pure Culture)
Gram Stain and cellular morphology
Gram-positive cocci
Perform catalase test
Catalase positive: Perform API for staphylococci/micrococci (API Staph)
Catalase negative: Perform API for streptococci/enterococci (API Strep)
Gram-positive bacilli
Perform API for Listeria (API Listeria) and for Bacillus (API 50CH)
Gram-negative cocci
Perform API for Neisseria Haemophilus/ Branhamella catarrhalis (API NH)
Oxidase positive: Perform API for non-Enterobacteriaceae (API 20NE)
Gram-negative bacilli
Perform oxidase test (dry slide format)
Oxidase negative: Perform API for Enterobacteriaceae and other non-fastidious Gram-negative bacteria (API 20E)

FIGURE 47.4 API Test Strip Chart

8 Based on the data you have thus far and considering the chart information, what is your next step (e.g., what API strip and/or test should you conduct)?

At the Patient's Bedside

The kidney stone was successfully removed. A temporary stent was placed in the ureter to keep it open as it heals from the trauma inflicted by the lodged stone. The urologist and infectious disease specialist are now concerned about the patient's infection and hope that by tomorrow the microbiological data will be ready.

In the Clinical Microbiology Lab

Day$_2$: Approximately 48 hours after Sample Collection

Today you are ready to analyze the biochemical test data to hopefully arrive at a definitive bacterial identification. You also will have some drug susceptibility data to send to the health-care team.

You retrieve the API test strip from the incubator, add reagents to the cupules per the manufacturer's specification, and observe the results in Figure 47.5.

Table 47.1 presents details for each test on the API strip you used to analyze the isolated bacterium. Unfortunately, your intern just dripped some reagents on the document and blurred a few areas. The good news is you have your lab manual from your undergraduate microbiology course available and your experience with biochemical tests, so you can fill in the few spaces where the information was blurred (numbers 9 through 13 in the table).

FIGURE **47.5** API Test Strip Results for Bacterium Isolated from Case Study Patient's Urine

TABLE **47.1** Results for Various Biochemical Tests

Test (Abbreviation)	Detects	Negative Result	Positive Result
ONPG	β-galactosidase (lactose hydrolysis)	Colorless	Yellow (pale color is possible)
ADH	Arginine dihydrolase	Yellow	Red or orange
LDC	Lysine decarboxylase	Yellow	Red or orange
ODC	Ornithine decarboxylase	Yellow	Red or orange
CIT	Citrate utilization	9.	10.
H_2S	Hydrogen sulfide (H_2S) production	Colorless	11.
URE	Urea hydrolysis	12.	13.
TDA	Tryptophan deamination	Yellow	Brown
IND	Indole production	Colorless/light yellow	Pink or red
VP	Acetoin production (Voges-Proskauer test)	Colorless	Pink or red (pale color is possible)
GEL	Gelatin hydrolysis	Colorless	Black (pigment diffuses)

(continues)

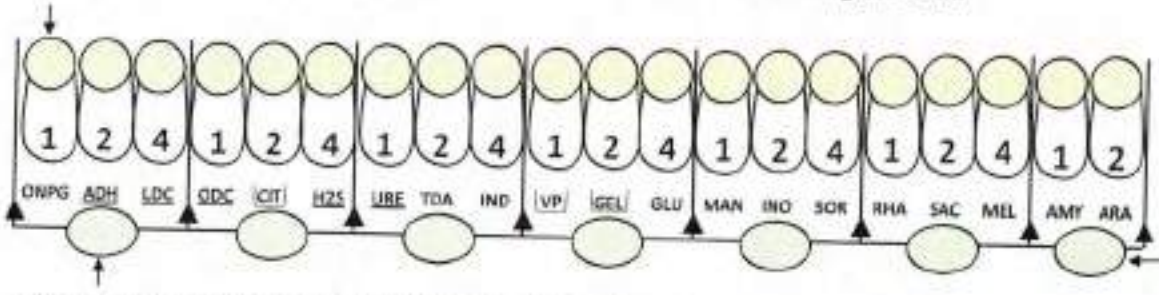
TABLE **47.1** *(continued)* **Results for Various Biochemical Tests**

Test (Abbreviation)	Detects	Negative Result	Positive Result
GLU	Glucose fermentation/oxidation	Blue	Yellow
MAN	Mannitol fermentation/oxidation	Blue	Yellow
INO	Inositol fermentation/oxidation	Blue	Yellow
SOR	Sorbitol fermentation/oxidation	Blue	Yellow
RHA	Rhamnose fermentation/oxidation	Blue	Yellow
SAC	Sucrose fermentation/oxidation	Blue	Yellow
MEL	Melibiose fermentation/oxidation	Blue	Yellow
AMY	Amagdalin fermentation/oxidation	Blue	Yellow
ARA	Arabinose fermentation/oxidation	Blue	Yellow

14 Finally, you're ready to analyze the test results and determine the bacterium's identity. Observe the API test results (Fig. 47.5) and use your completed table (Table 47.1) to fill out the following data recording sheet.

API RESULTS Sample Origin: _____ Date Inoculated: _____

Step 1: In each yellow circle, write a "+" for each positive result and a "–" for each negative result.



If the oxidase test was positive, add 4 to this last number. Note, the oxidase test is not on the test strip, but counts toward the final ID number.

Step 2: Add the numbers for the *positive* (+) test results for each section and write the sum in each green oval. For example, if all three tests in the first section (ONPG, ADH, and LDC) were positive, you'd write a "7" in the first green oval. If only ODC and H2S were positive for the second section, you'd write a "5" in the second green oval. Do this until each green oval has a numerical value. If none of the tests in a section are positive, enter a "0" for the section.

Step 3: To determine the bacterium's identity, look at Table 47.2 and find the 7-digit numerical sequence generated by the green ovals.

Step 4: Bacterium's most likely identity: _____

TABLE **47.2** API Identification Table* •

Bacterium	ID number	Bacterium	ID number
Xanthomonas axonopodis	0001000	*Pantoea ananatis*	1245573
Erwinia tracheiphila	0004520	*Pasteurella haemolytica*	1422000
Erwinia amylovora	0005020	*Stenotrophomonas maltophilia*	1422351
Morganella morganii	0174000	*Citrobacter freundii*	1604573
Pseudomonas solanacearum	0201004	*Pseudomonas aeruginosa*	2214006
Erwinia salicis	0204731	*Pasteurella multocida*	3000004
Pseudomonas rubrilineans	0211004	*Vibrio hollisae*	3042140
Ablotrophia adiacens	0402000	*Enterobacter cloacae*	3305773
Streptococcus acidominimus	0470000	*Enterobacter agglomerans*	3307173
Proteus mirabilis	0736000	*Aeromonas hydrophila /caviae*	3577754
Xanthomonas albilineans	1001000	*Hafnia alvei*	4105112
Escherichia adecarboxylata	1004173	*Edwardsiella tarda*	4744000
Erwinia stewartii	1005162	*Streptococcus mutans*	5040710
Corynebacterium fasciam	1010000	*Escherichia coli*	5144572
Agrobacterium tumefaciens	1010007	*Klebsiella pneumoniae*	5215773
Agrobacterium radiobacter	1015046	*Serratia marcescens*	5306563
Pasteurella pneumotropica	1020300	*Chryseomonas luteola*	5477761
Bordetella branchiseptica	1200063	*Aerococcus viridans*	6112711
Serratia rubidaea	1207360	*Salmonella choleraesuis*	6704510
Erwinia chrysantherni	1244173	*Vibrio parahaemolyticus*	7467746

* These API codes are simply examples, they are for teaching purposes only and do not necessarily represent the most recent API code data maintained in the subscription-based API database.

At the Patient's Bedside

The infectious disease doctor reported the microbiology findings to the rest of the health-care team. Based on the microbiology data, the bacterium proved susceptible to several common oral antibiotics. The care providers agreed that the patient could be discharged from the hospital and sent home with a three-day course of ciprofloxacin. The patient fully recovered, although he did experience two more kidney infections over the next 5 years.

15 Look up the bacterium that you identified as the causative agent and describe some of the features that can make it a challenging pathogen to combat.

EXERCISE 48

Bacterial Unknown Exercise and Resources

After doing the unknown exercise students typically are able to:

1. Perform various staining protocols and biochemical tests on a bacterial unknown sample.
2. Use a light microscope to make morphological and staining observations.
3. Record and analyze observations for staining and biochemical tests to discover the identity of an unknown bacterial sample.
4. Report observations and conclusions for a variety of staining procedures and biochemical tests.

Background

A capstone laboratory experience in most undergraduate microbiology courses is the identification of a bacterial unknown. While the approach your instructor uses for this sort of exercise may vary, the general principle is the same: the student applies their newly developed microbiology lab skills to determine the identity of a bacterial sample. The sample may be provided to you as a pure culture or as a mixed culture.

Most instructors have students perform this lab once the class has some experience with staining methods and students have learned how to perform and interpret a variety of biochemical tests. In some courses, you may complete an unknown project at a midpoint in the course and then a more sophisticated unknown project closer to the end of the course. In other courses, you may simply have one unknown project that serves as a laboratory capstone experience.

What to Expect During an Unknown Exercise

You can expect to perform a variety of biochemical tests and stains on a bacterial specimen issued to you and then use the data from those procedures to decide your bacterium's identity. Typically, this exercise is done over two or more lab sessions. You should not expect much guidance from your instructor; rather this is *your* opportunity to solve a real-world problem with your own acquired skills. You've got great new skills and now is your chance to show them off!

In most cases, the better you are at implementing the lab techniques you explored in your course, the more likely you are to correctly identify your bacterial specimen's identity. However, correctly identifying your bacterium's identity is often just one part of your score. Usually your instructor will be looking at your lab writeup or report to see if you truly know how to perform and interpret a variety of biochemical tests and staining protocols. They will also be checking that you are detail-oriented and thorough in terms of how you report your observations and formulate conclusions. And of course, you are always expected to use the proper language and writing conventions for your documentation.

Useful Resources

Refer to the notecards at the end of the book, you'll likely find them useful as you undertake this exercise. These notecards can also be cut out and used as a study resource! Additional resources that you'll likely find useful for this exercise include the Instructional Staining Atlas (pp. 131–135) along with the identification flowcharts in Appendices F–I (pp. 487–500).

MATERIALS

- ❑ Lab coat
- ❑ Gloves
- ❑ Safety glasses
- ❑ Unknown mixture with number (1, your instructor will advise you whether the sample is pure or mixed)
- ❑ Appropriate stains
- ❑ Appropriate biochemical test media and reagents
- ❑ Recommended organisms (samples may be mixed or pure as your instructor decides)

Gram-Positive Candidates

- ☐ *Bacillus cereus*
- ☐ *Bacillus coagulans*
- ☐ *Bacillus subtilis*
- ☐ *Corynebacterium xerosis*
- ☐ *Enterococcus faecalis* (BSL-2)
- ☐ *Kocuria rosea*
- ☐ *Lactobacillus plantarum*
- ☐ *Micrococcus luteus*
- ☐ *Mycobacterium smegmatis* (BSL-2)
- ☐ *Staphylococcus aureus* (BSL-2)
- ☐ *Staphylococcus epidermidis*
- ☐ *Streptococcus salivarius*

(continues)

MATERIALS *(continued)*

Gram-Negative Candidates

- ☐ *Citrobacter freundii*
- ☐ *Enterobacter aerogenes* (BSL-2)
- ☐ *Enterobacter cloacae*
- ☐ *Escherichia coli*
- ☐ *Hafnia alvei*
- ☐ *Klebsiella pneumoniae* (BSL-2)
- ☐ *Morganella morganii* (BSL-2)
- ☐ *Proteus mirabilis* (BSL-2)
- ☐ *Proteus vulgaris* (BSL-2)
- ☐ *Providencia stuartii*
- ☐ *Serratia liquefaciens*
- ☐ *Serratia marcescens*

Other bacteria as decided by your instructor

- ☐ _____
- ☐ _____
- ☐ _____

9

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 48 DATA SHEET

PROCEDURE

Unknown Report

Unknown Sample # _____

My instructor wants us to work in a group of: _____ (You'll usually work either alone or in a group of two, but this is at your instructor's discretion.)

CAUTION!

Unless your instructor has specified otherwise, assume your sample could be a BSL-2 bacterium and follow all BSL-2 precautions.

STEP 1: OBTAIN A PURE CULTURE AND NOTE COLONY FEATURES

The provided unknown culture is: mixed/pure (select one).
If you are given a mixed culture, your instructor will *likely* tell you how many bacteria are in the sample, you should take note of that information. If you have a mixed culture, then you will need to perform a streak plate isolation to detect the constituents.

Observe the culture characteristics of your pure culture.
Once you have a pure culture with isolated colonies, report features such as the color, shape, elevation, shine or no shine, and borders of the colonies.

Write your observations here (refer to Ex. 6 for help).

Size: _____

Shape (form): _____

Margin (edge): _____

Elevation: _____

Consistency: _____

Surface Texture: _____

Other useful features (e.g., color, optical properties): _____

STEP 2: PERFORM STAINS ON YOUR PURE CULTURE AND MAKE OBSERVATIONS

Place a checkmark next to the stains your instructor wants you to perform and write your observations and conclusions in the following table for each stain you perform.

Stain	Observations
Simple (See Ex. 11)	
Negative (See Ex. 12)	
Gram (See Ex. 13)	
Acid-Fast (See Ex. 14)	
Capsule Stain (See Ex. 15)	
Endospore Stain (See Ex. 16)	

Based on your observations, write a final description of the cellular morphology and specific structures and/or cell wall properties of your bacterium:

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 48 DATA SHEET

(continued)

STEP 3: PERFORM BIOCHEMICAL TESTS ON YOUR PURE CULTURE AND MAKE OBSERVATIONS

Place a checkmark next to the plated media and various biochemical tests your instructor will have available for you to assess your sample.

Differential/Selective Media

On pages 441–443, you will find four summary notecards of differential and selective media. These can be cut out and mounted onto 4 × 6 or 5 × 8 notecards as a reference tool during the lab.

Media	Observations	Conclusions
Mannitol Salt Agar (MSA) (See Ex. 22)		
MacConkey (MAC) Agar (See Ex. 23)		
Eosin Methylene Blue (EMB) Agar (See Ex. 24)		
Blood Agar (See Ex. 25)		

Biochemical Tests

On pages 445–461, you will find 13 summary notecards of biochemical tests. These can be cut out and mounted onto 4 × 6 or 5 × 8 notecards, or other cardstock as a reference tool during the lab.

Test	Observations	Conclusions
Glucose (Dextrose) Fermentation Phenol Red Broth (See Ex. 27)		
Lactose Fermentation Phenol Red Broth (See Ex. 27)		
Sucrose Fermentation Phenol Red Broth (See Ex. 27)		
Methyl Red and Voges-Proskauer (MR-VP) Tests (See Ex. 28)	MR: VP:	MR: VP:
Catalase Test (See Ex. 29)		
Oxidase Test (See Ex. 30)		
Nitrate Reduction Test (See Ex. 31)		
Citrate Utilization (See Ex. 32)		
Lysine Amino Acid Decarboxylation (Decarboxylase Tests) (See Ex. 33)		
Ornithine Amino Acid Decarboxylation (Decarboxylase Tests) (See Ex. 33)		
Arginine Amino Acid Decarboxylation (Decarboxylase Tests) (See Ex. 33)		

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 48
DATA SHEET
(continued)

Test	Observations	Conclusions
Starch Hydrolysis (Amylase Test) (See Ex. 34)		
Urea Hydrolysis (Urease Test) (See Ex. 35)		
SIM Medium: Sulfur, Indole, and Motility Tests (See Ex. 36)	H_2S: Indole: Motility:	H_2S: Indole: Motility:
Triple Sugar Iron Agar/Kligler Iron Agar (See Ex. 37)		
Coagulase Test (See Ex. 38)		
Gelatin Hydrolysis (Gelatinase Test) (See Ex. 39)		

OTHER TESTS

Test name	Observations	Conclusions

STEP 4: USE YOUR DATA TO DETERMINE THE BACTERIUM'S IDENTITY

Use the flow charts in Appendices F–I (pp. 487–500) to determine which bacterium you most likely have. Note that you are unlikely to have a perfect match, your task is to find the best match.

Bacterium's Identity: _____ (include genus and species)

STEP 5: REFLECTIVE ANALYSIS

1 What test results for this exercise did *not* align with your conclusion about the bacterium's identity?

2 Regarding the data you stated were incongruent with your conclusion, explain what may have gone wrong.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 48
DATA SHEET
(continued)

3 Provide some background about the bacterium you identified. Include the following in your discussion:

a. Morphological features

b. Gram property

c. Places the bacterium can naturally be found

d. Clinical, environmental, and/or industrial significance of the bacterium

e. Three additional interesting facts about the bacterium

— Lab Notes —

A Summarized Guide of Differential/ Selective Media for the Identification of an Unknown Bacterial Sample

Notecards

Mannitol Salt Agar (MSA) (From Ex. 22)

Purpose: This media is selective for salt-tolerant organisms such as *Staphylococcus, Bacillus, Enterococcus, Micrococcus,* and others. MSA also contains the sugar mannitol, so those bacteria that grow on the media can be differentiated based on their ability to ferment mannitol.

How to Inoculate: Use an inoculating loop to perform a streak plate isolation or T-streak of the plated media.

Incubation Parameters: Invert the plate and incubate all the samples at 35±2°C for 48 hours.

Interpretations: After incubation, note if the bacterium grew or not. If you observe growth, then assess the media's color around the colonies.

Results	Interpretations
Poor growth (P) or no growth (N)	The organism's growth is inhibited by NaCl; the bacterium is unlikely to be a *Staphylococcus* species.
Good growth (G)	The organism's growth is not inhibited by NaCl. It's possible the bacterium is a *Staphylococcus* species, or some other bacterium known to grow well on MSA (e.g., a *Bacillus, Enterococcus, Micrococcus* species).
Yellow growth or halo (Y)	The organism fermented mannitol; the pH was lowered by acids generated during mannitol fermentation. The bacterium may be pathogenic *Staphylococcus aureus* (further testing needed to make this conclusion; many *Staphylococcus* species ferment mannitol and some ferment none; *Staphylococcus* bacteria may ferment mannitol).
Red growth (no halo) (R)	The organism does not ferment mannitol. The organism is unlikely to be *S. aureus*.

MacConkey (MAC) Agar (From Ex. 23)

Purpose: This media is selective for Gram-negative bacteria and selective based on the fermentation of lactose. MAC is one example of an enriched selective and differential growth media used for detecting coliforms (to include *E. coli*) in a sample.

How to Inoculate: Use an inoculating loop to perform a streak plate isolation or T-streak of the plated media.

Incubation Parameters: Invert the plate and incubate all the samples at 35±2°C for 48 hours.

Interpretations: After incubation, note if the bacterium grew or not. If you observe growth, then assess the bacterial growth's color.

Results	Interpretations
Poor growth (P) or no growth (N)	Gram-positive bacterium
Good growth (G)	Gram-negative bacterium
Pink to red growth with or without bile precipitate (R)	The organism produces acid as a byproduct of lactose fermentation; the bacterium may be a coliform bacterium.
Growth is "colorless" (not red or pink) (C)	The organism does not ferment lactose; it is unlikely to be a coliform.

Eosin Methylene Blue (EMB) Agar (From Ex. 24)

Purpose: EMB is an enriched selective and differential growth media used for detecting coliforms (to include *E. coli*) in a sample. Like MAC agar, EMB is selective for Gram-negative bacteria and differential based on the fermentation of lactose.

How to Inoculate: Use an inoculating loop to perform a streak plate isolation or T-streak of the plated media.

Incubation Parameters: Invert the plate and incubate all the samples at 35 ± 2°C for 48 hours.

Interpretations: After incubation, note if the bacterium grew or not. If you observe growth, then assess the bacterial growth's color.

Results	Interpretations
Poor growth (P) or no growth (N)	Gram-positive bacterium
Good growth (G)	Gram-negative bacterium
Growth is pink and mucoid (Pi)	The organism produces acid as a byproduct of lactose fermentation and is possibly a coliform bacterium.
Growth is "dark" (purple to black, with or without green metallic sheen) (D)	The organism produces acid as a byproduct of lactose fermentation and is probably a coliform bacterium.
Growth is "colorless" (not purple, green, red, or pink) (C) (*Note:* EMB media has a reddish color so do not mistake the color of the media as being the color of the growth.)	The organism does not ferment lactose and is unlikely to be a coliform bacterium.

Blood Agar (From Ex. 25)

Purpose: Blood agar detects hemolytic activity and is commonly used to differentiate β-hemolytic, pathogenic *Streptococcus* species from non-pathogenic species of this diverse genus of Gram-positive cocci.

How to Inoculate: Use an inoculating loop to perform a streak plate isolation or T-streak of the plated media.

Incubation Parameters: Invert the plate and incubate your sample at 35 ± 2°C for 24 hours.

Interpretations: After incubation, assess the color of the media around the bacterial growth.

Results	Interpretations
Clearing around growth	The organism hemolyzes RBCs completely (β-hemolysis).
Greening around growth	The organism partially hemolyzes RBCs; hemoglobin oxidized to methemoglobin (α-hemolysis).
No change in the medium	The organism does not hemolyze RBCs (γ-hemolysis).

A Summarized Guide of Biochemical Tests for the Identification of an Unknown Bacterial Sample

Notecards

Phenol Red Fermentation Broth: Glucose, Lactose, and Sucrose Fermentation Tests (From Ex. 27)

Purpose: These tests detect the enzymes needed to ferment a given carbohydrate. If a given carbohydrate is fermented, then acids are made that will lower the media's pH. As pH drops, the pH indicator in the media (phenol red) turns from its original red color to yellow. If the bacteria do not ferment the given carbohydrate, then the media remains red. If gas is produced as a by-product of fermentation, then the Durham tube will have a bubble in it.

How to Inoculate: All carbohydrate test media should be inoculated with the transfer loop.

Incubation Parameters: Incubate the sample at 35 ± 2°C for 48 hours.

Interpretations: When making interpretations, make sure you note media coloration as well as note if gas is present or absent in the Durham tube.

Results	Interpretations
Yellow broth; bubble in Durham tube	The carbohydrate (insert sugar's name) was fermented and acidic end products were formed; gas was also produced as a byproduct. If lactose was fermented, you can note that your test bacterium makes the enzyme β-galactosidase; if sucrose was fermented, you can state your test bacterium makes sucrase. Note the result as A/G.
Yellow broth; no bubble in Durham tube	The carbohydrate (insert sugar's name) was fermented and acidic end products were formed; gas was also produced as a byproduct. If lactose was fermented, you can note that your test bacterium makes the enzyme β-galactosidase; if sucrose was fermented, you can state your test bacterium makes sucrase. Note the result as A/–.
Red broth; no bubble in Durham tube	The carbohydrate (insert sugar's name) was not fermented and gas was not produced. Note the result as –/–.
Pink broth; no bubble in Durham tube	Degradation of peptone occurred to form alkaline end products, but the carbohydrate (insert sugar's name) was not fermented; gas was not produced. Note the result as K/–.

Methyl Red and Voges-Proskauer (MR-VP) Tests (From Ex. 28)

Purpose: The methyl red and Voges-Proskauer tests (MR-VP) help us differentiate bacterial species based on the fermentation pathway used to metabolize glucose. The MR portion of the test detects if mixed acid fermentation occurred. The Voges-Proskauer test reveals if a bacterium metabolized glucose via the butanediol fermentation pathway.

How to Inoculate: Inoculate the MR-VP broth with a transfer loop.

Incubation Parameters: Incubate the sample at 35 ± 2°C for 48 hours.

Test Development Process: For this test, only one tube of media is inoculated. After incubation, 1 mL of the culture is transferred to a clean test tube to serve as the MR portion of the test and another 1 mL of culture is transferred to a tube and analyzed for the VP portion of the test. Follow the development protocols in the following tables for the MR and VP test, respectively.

Methyl Red Test

- Add 3 drops of methyl red reagent to the sample and immediately observe for the development of a red color.

Results	Interpretations
Red	Organism performs a mixed acid fermentation. Note this as a positive result (+).
No color change	Organism does not perform a mixed acid fermentation; acidic end products were not made in detectable amounts. Note this as a negative result (–).

Voges-Proskauer Test

- Add 15 drops (0.6 mL) of VP Reagent A. Mix well.
- Add 5 drops (0.2 mL) of VP Reagent B. Mix well.
- Observe for red color formation at 10-minute intervals for up to 1 hour. Periodically mix the sample.

Results	Interpretations
Red	Organism performs a 2,3-butanediol fermentation (acetoin was produced). Note this as a positive result (+).
No color change	Organism does not perform a 2,3-butanediol fermentation (acetoin was not produced or it is not detectable). Note this as a negative result (–).

Catalase Test (From Ex. 29)

Purpose: This test detects catalase, an enzyme responsible for protecting bacteria from hydrogen peroxide (H_2O_2) accumulation, which can occur during aerobic metabolism. If hydrogen peroxide accumulates, it becomes toxic to the organism. Catalase converts H_2O_2 to water and gaseous oxygen.

Test Development Process: Test fresh (18- to 24-hour) agar slant cultures. Inoculation and incubation are not required. This test is often performed by adding hydrogen peroxide to a bacterial smear on a microscope slide. If using this "slide method," cover the sample after adding hydrogen peroxide so that you reduce workspace contamination from the aerosols a positive test may generate.

Another way to perform the catalase test is to add hydrogen peroxide directly to bacteria growing on an agar slant or agar plate (do not use cultures growing on blood agar or you will likely get a false-positive result). Adding H_2O_2 directly to the culture will render the sample unusable for further tests. If you need the culture for further testing, then use the slide method for this test.

Results	Interpretations
Bubbles immediately develop	Catalase is present; the organism is aerobic. Note this as a positive result (+).
No bubbles detected	Catalase is absent or not detectable. (**Note:** You *cannot* conclude the bacterium is anaerobic just because it lacks catalase!) Note this as a negative result (–).

Oxidase Test (From Ex. 30)

Purpose: The oxidase test reveals if a bacterium has cytochrome c oxidase, an enzyme in certain aerobic electron transport chains. Bacteria that have cytochrome c oxidase respire aerobically. However, a negative oxidase test result only means the bacterium lacks cytochrome c oxidase; it does not necessarily mean the bacteria cannot respire aerobically using some other ETC that doesn't require cytochrome c oxidase.

Test Development Process: There are several ways to perform the oxidase test:

1. Add the TMPD reagent directly to bacterial growth on solid media.
2. Smear the test bacterium onto filter paper and then add TMPD to the smear.
3. Smear the test bacterium onto paper infused with the TMPD reagent. (Commercially available DrySlide.)

Regardless of the approach applied, if cytochrome c oxidase is present a color change to purple or blue occurs within seconds as TMPD is oxidized.

Results	Interpretations
Culture DrySlide **Dark blue/purple color develops within 20 seconds**	Cytochrome c oxidase is present; the organism can perform aerobic respiration. Note this as a positive result (+).
Culture DrySlide **No color change to blue/purple within 20 seconds**	Cytochrome c oxidase is not present or not detectable. (**Note:** Do *not* assume the tested bacterium is anaerobic; the bacterium may have alternative aerobic ETCs that do not require cytochrome c oxidase.) Note this as a negative result (–).

Nitrate Reduction Test (From Ex. 31)

Purpose: The nitrate test allows us to differentiate bacterial species from one another based on their ability to reduce nitrate (NO_3^-). An inverted Durham tube in each broth serves to trap a portion of any produced gas.

How to Inoculate: Inoculate nitrate broth with a transfer loop.

Incubation Parameters: Incubate the sample at 35 ± 2°C for 24 to 48 hours.

Test Development Process: Following incubation, analyze the test via a three-step process.

1. **Check for a Bubble in the Durham Tube:** If there is a bubble *and* you are certain the bacterium does *not* conduct fermentation, then you may safely conclude the tested organism reduces nitrate to gaseous end products such as molecular nitrogen (N_2) or nitrous oxide (N_2O). In most cases, you will need to move to Step 2. (See table Development Process 1.)
2. **Add Reagents A and B:** If you are *not* certain as to whether your bacterium conducts fermentation or not, then you must add various reagents to the sample. Add 8 drops (approximately 0.5 mL) of Reagent A and then 8 drops (approximately 0.5 mL) of Reagent B to the tube and mix well. If you see a red color after adding Reagents A and B, then you may conclude the tested bacterium reduces nitrate to nitrite. (See table Development Process 2.)
3. **Add Reagent C if Red Color Does NOT Develop During Step 2 Above:** If you do *not* see a color change to red after adding Reagents A and B in Step 2, then add a small amount of nitrate Reagent C (zinc dust) to determine if the bacterium reduced nitrate beyond nitrite. You can dip a wooden applicator into the zinc dust and transfer the amount that clings to the wood. Let the tubes stand for 10 minutes. If a red color develops, then the organism did *not* reduce nitrate. Lack of a color change after adding Reagent C indicates the organism reduced nitrate to NH_4^+, NO, N_2O, or some other nitrogenous compound other than nitrite. (See Development Process 3 in the following table.)

Development Process	Results	Interpretations
1. Check for a Bubble in the Durham Tube.	Gas in Durham tube (no reagents added). (*Note:* Only use this measure if you are certain the tested bacterium does *not* conduct fermentation. If you do not know the fermentation status of the bacterium, proceed to Development Process 2.)	If you are certain the test bacterium does *not* perform fermentation, then you may conclude denitrification occurred ($NO_3^- \rightarrow NO_2^- \rightarrow N_2$). Note this as a positive result (+).
2. Add Reagents A and B.	Red color after adding Reagents A and B. (The test is completed; no further reagents should be added.)	Nitrate reduction to nitrite ($NO_3^- \rightarrow NO_2^-$); nitrate reductase is present. Note this as a positive result (+).
	No color change after adding Reagents A and B. (Proceed to Development Process 3.)	Incomplete test; requires the addition of zinc dust. (A test result cannot be determined yet. Proceed to adding Reagent C, Development Process 3.)
3. Add Reagent C if Red Color Does NOT Develop During Step 2.	No color change after adding Reagent C. (The test is completed; no further reagents should be added.)	Nitrate reduction to gaseous (NO, ½N_2O, or ½N_2) or reduction to nongaseous nitrogen compounds such as ammonium (NH_4^+) occurred. Note this as a positive result (+).
	Red color after adding Reagent C. (The test is completed; no further reagents should be added.)	No nitrate reduction. Note this as a negative result (–).

Citrate Utilization Test (From Ex. 32)

Purpose: This biochemical test detects the ability of bacteria to use citrate as a sole carbon source. If the bacterium cannot use citrate as its sole carbon source, then it will not grow and the media will remain a greenish color. If the bacterium can use citrate as its sole carbon source, then the bacteria will grow and the media will turn a bright blue as a result of an increase in the pH of the media.

How to Inoculate: Use an inoculating loop to streak the bacteria onto citrate media in a zigzag fashion along the slant surface ("fishtail inoculation"). To avoid confusion between actual growth and a heavy inoculum, inoculate lightly.

Incubation Parameters: Incubate the sample at 35 ± 2°C for 48 hours.

Results		Interpretations
	Media appears blue (even if only a portion of the media turned blue) and growth is evident on the slant surface.	Citrate is utilized (organism has the enzymes needed to use citrate as its sole carbon source). Note this as a positive result (+).
	No color change, but growth is evident on the slant surface.	Citrate is utilized (organism has the enzymes needed to use citrate as its sole carbon source). Note this as a positive result (+).
	No color change and no growth on the slant surface.	Citrate is not utilized (organism lacks one or more of the enzymes needed to use citrate as its sole carbon source). Note this as a negative result (–).

Lysine/Ornithine/Arginine Amino Acid Decarboxylation (Decarboxylase Tests) (From Ex. 33)

Purpose: This biochemical test allows us to examine the ability of bacteria to decarboxylate the amino acid lysine using the enzyme lysine decarboxylase. As optional procedures, you may also test for the presence of ornithine decarboxylase and arginine decarboxylase in the assigned bacteria. (*Note:* Each decarboxylase is specific to its own amino acid substrate.)

How to Inoculate: Use an inoculating loop to inoculate the broth decarboxylase media. After inoculation, an overlay of mineral oil is added to seal the medium from the air. This ensures the surface of the media's pH is not artificially raised (alkalized) through interactions with the ambient air and it helps to maintain a lower oxygen environment that favors glucose fermentation.

Incubation Parameters: Incubate the sample at 35 ± 2°C for 96 hours (4 days) up to a full week.

Results		Interpretations
	No color change	No decarboxylation of the amino acid (insert amino acid name)*; the specific decarboxylase is absent or products are not detectable. Note this as a negative result (–).
	Yellow	Fermentation of glucose; no decarboxylation of the amino acid (insert amino acid name)*; the specific decarboxylase is absent or products are not detectable. Note this as a negative result (–).
	Purple (may be slight)	Decarboxylation of the amino acid (insert amino acid name)*; organism produces the specific decarboxylase enzyme. Note this as a positive result (+).

* The amino acid included in the media can vary. Therefore, describe all interpretations as they relate to the specific amino acid in the media. Example: if using lysine decarboxylase media, the amino acid in the media is lysine.

Starch Hydrolysis (Amylase Test) (From Ex. 34)

Purpose: The starch hydrolysis test (or amylase test) reveals if a bacterium can break down the complex polysaccharide starch.

How to Inoculate: Use an inoculating loop to inoculate starch agar with the test bacterium. You may use a streak plate isolation method, or if you have a pure culture, you may use a zigzag inoculation.

Incubation Parameters: Incubate the sample at 35 ± 2°C for 24 hours.

Test Development Process: After incubation, cover the growth and surrounding areas with Gram's iodine. Do not flood the plate but do add enough iodine so that the agar is completely colorized. *Allow the iodine to soak in.* After the agar has soaked up the iodine, check for a clear zone around the bacterial growth.

Results		Interpretations
	A clearing around the growth is evident after adding iodine to the plate.	α-amylase, β-amylase, and/or α-1,6-glucosidase is present (or more simply, amylase is present). Note this as a positive result (+).
	There is not a clearing around the growth after adding iodine to the plate.	Fermentation of glucose; no decarboxylation of the amino acid (insert amino acid name); the specific decarboxylase is absent or products are not detectable. Note this as a negative result (–).

Urea Hydrolysis (Urease Test) (From Ex. 35)

Purpose: This test reveals if a bacterium produces the intracellular hydrolytic enzyme urease, which uses water to catabolize (break down) urea to form ammonia and carbon dioxide.

How to Inoculate: Use an inoculating loop to inoculate urea media.

Incubation Parameters: Incubate the sample at 35 ± 2°C for 24 hours.

Results		Interpretations
	Orange or yellow	No urea hydrolysis; urease is absent or not produced in enough quantity to produce results in 24 hours; or the organism cannot live in urease broth. Note this as a negative result (–).
	Pink	Rapid urea hydrolysis; urease was produced. Note this as a positive result (+).

SIM Medium (Sulfur, Indole, and Motility Tests) (From Ex. 36)

Purpose: This media assesses a bacterium's ability to reduce sulfur, make indole, and move.

How to Inoculate: Use an inoculating needle to stab the SIM media in as straight a line as possible through the middle of the agar deep. Insert the needle into the agar to within 1 cm of the bottom of the tube. Be careful to remove the needle along the original stab line so that you obtain a straight inoculation.

Incubation Parameters: Incubate the sample at 35 ± 2°C for 24 to 48 hours.

Sulfur Reduction (H_2S Production)—The "S" in SIM

- In addition to SIM media, TSI (triple sugar iron) media can also be used to assess sulfur reduction.

Results	Interpretations
No black in the medium	Sulfur is not reduced, or is not reduced in detectable amounts. Note this as a negative result (–).
Black in the medium	Sulfur reduction occurred (H_2S was made); organism either respires anaerobically with sulfate as the final electron acceptor (thiosulfate reductase) or hydrolyzes the amino acid cysteine (cysteine desulfurase). Note this as a positive result (+).

Indole Production—The "I" in SIM

- Add Kovac's reagent to each SIM media tube (including the control) to a depth of 2 mm–3 mm (*not mL*). After several minutes, observe for the formation of red color in the reagent layer.

Results	Interpretations
No red layer evident after adding Kovac's reagent	Tryptophanase is absent and indole is not produced, or it's produced but not in detectable amounts. Note this as a negative result (–).
Red layer evident after adding Kovac's reagent	The organism produces tryptophanase and hydrolyzes tryptophan into indole, NH_3, and pyruvate. Note this as a positive result (+).

Motility—The "M" in SIM

- In addition to SIM media, general motility agar may be used to assess motility. Unlike SIM media, general motility agar only reveals information about a bacterium's motility.

Results	Interpretations
No growth radiating away from the original inoculation/ stab line.	Nonmotile. Note this as a negative result (–).
Growth radiating outward from inoculation/stab line.	Motile. Note this as a positive result (+).
Blackened medium	Motility cannot be determined; use general motility agar to assess motility. See Exercise 36 for details on general motility agar.

Triple Sugar Iron Agar (TSIA)/Kligler Iron Agar (KIA) (From Ex. 37)

Purpose: Differentiates bacteria based on sulfur reduction and sugar fermentation.

How to Inoculate: Both TSIA and KIA are prepared as a shallow agar slant with a deep butt. This format provides both aerobic and anaerobic growth environments. Both media are double inoculated; first use an inoculating needle to stab the butt of the agar and then use a loop to zigzag inoculate the slant ("fishtail inoculation").

Incubation Parameters: For carbohydrate fermentation assessment, incubate the sample at 35 ± 2°C for 18–24 hours. After interpreting the carbohydrate fermentation results, samples are re-incubated for an additional 24 hours at 35 ± 2°C before making a final H_2S determination.

Results	Interpretations
Yellow slant/yellow butt—KIA	KIA: Glucose **and** lactose fermentation with acid accumulation in slant and butt. Note this test result as A/A.
	TSIA: Glucose and lactose **and/or** sucrose fermentation with acid accumulation in slant and butt. Note this test result as A/A.
Red slant/yellow butt	TSIA and KIA: Glucose fermentation with acid production; proteins catabolized aerobically (in the slant) with alkaline products (reversion). Note this test result as K/A.
Red slant/red butt	TSIA and KIA: No fermentation; peptone catabolized aerobically and anaerobically with alkaline products; isolate is not from *Enterobacteriaceae*. Note this test result as K/K.
Red slant/no change in the butt	TSIA and KIA: No fermentation; peptone catabolized aerobically with alkaline products; isolate is not from *Enterobacteriaceae*. Note this test result as K/NC.
No change in slant/no change in butt	TSIA and KIA: Organism is growing slowly or not at all; isolate is not from *Enterobacteriaceae*. Note this test result as NC/NC.
Black precipitate in the agar	TSIA and KIA: Sulfur reduction (an acidic condition from glucose fermentation exists in the butt even if the yellow color is obscured by the black precipitate). Note this test result as H_2S made.
Cracks in or lifting of agar	TSIA and KIA: Gas production. Note this test result as G.

Coagulase Test (From Ex. 38)

Purpose: Differentiates bacteria based on the presence of coagulase, an enzyme that most pathogenic *Staphylococcus aureus* make. The coagulase test differentiates coagulase-positive *Staphylococcus aureus* from other, usually less virulent, coagulase-negative *Staphylococcus* species.

How to Inoculate: Use a sterile wooden applicator or disposable loop to inoculate a tube of rabbit plasma plus EDTA with a pure culture of the test bacterium. Mix the contents by gently rolling the inoculated tube between your hands.

Incubation Parameters: Incubate all tubes at 35 ± 2°C for up to 24 hours, checking for coagulation periodically for the first 4 hours or remainder of your lab. Do not exceed 24 hours of incubation.

Interpretations: Tilt media to see if it solidified.

Results	Interpretations
Medium solidified within 24 hours or there is evident clumping/ coagulation (even if the media did not entirely solidify).	Free or bound coagulase present; plasma coagulated; probable *Staphylococcus aureus*. Note this as a positive result (+).
Medium remains liquid after 24 hours; no signs of coagulation are evident.	Plasma did not coagulate; coagulase was not detectable; unlikely to be *S. aureus*. Note this as a negative result (–).

Gelatin Hydrolysis (Gelatinase Test) (From Ex. 39)

Purpose: To detect the enzyme gelatinase, which breaks down gelatin into amino acids.

How to Inoculate: Use an inoculating needle to stab a tube of nutrient gelatin media. Use a heavy inoculum of 18- to 24-hour-old sample.

Incubation Parameters: Two incubation parameters are acceptable. Either incubate at 25 ± 2°C for up to 2 weeks or at 35 ± 2°C for up to 1 week.

Interpretations: Tilt the media to see if it solidified. *Note:* If you incubated the samples at 35°C, then you must place the samples (to include an uninoculated control that was incubated alongside the test sample) in an ice bath or refrigerator for about 15 minutes (or until the uninoculated control solidifies).

Results	Interpretations
Medium remains solid after refrigeration.	The organism does not make the enzyme gelatinase. Note this as a negative result (–).
Medium remains liquid after refrigeration.	The organism makes the enzyme gelatinase. Note this as a negative result (+).

Appendices

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— Lab Notes —

APPENDIX A
Biochemical Pathways

A lot of what we do in microbiology relates back to basic biochemical pathways (Fig. A.1). The discussion and figures in this appendix illustrate how the various biochemical tests presented in this manual fit into the overall biochemical scheme.

Oxidation of Glucose: Glycolysis, Entner-Doudoroff, and Pentose-Phosphate Pathways

Most organisms perform glycolysis (Embden-Meyerhof-Parnas pathway) (Fig. A.2). Glycolysis breaks glucose into two molecules of pyruvate and, in the process, releases some energy. The energy-releasing (exergonic) reactions that generate ATP do so via a process called **substrate phosphorylation**. Although glycolysis yields a total of four ATP, two ATP are invested early in the pathway, leaving the cell with a net production (profit) of two ATP for each glucose molecule that enters glycolysis.

Glycolysis also generates reducing power—namely NADH—that is important for a variety of reactions to include the production of ATP in electron transport chains by **oxidative phosphorylation**. If environmental conditions do not favor the operation of electron transport chains (e.g., a necessary final electron acceptor is absent), then many cells can funnel NADH into fermentation pathways. Although fermentation pathways do not usually make ATP[1], they do help to regenerate NAD^+ (a necessary cofactor to oxidize glucose) so that glycolysis can continue to make some ATP for cell survival. In summary, each glucose that enters glycolysis will generate two pyruvate molecules, two NADH, and two net molecules of ATP (Table A.1).

In addition to metabolizing sugars, glycolysis is a sort of "metabolic crossroad" that supports anabolism (building) and catabolism (break down) of other biomolecules. Glycolytic intermediates serve as building blocks of certain amino acids, lipids, and nucleotides. The carbon skeletons of these same biomolecules can also be funneled into glycolysis and broken down. Some of these anabolism/catabolism linkages are shown in Figure A.1.

[1] The acetate fermentation pathway does generate some ATP.

The Entner-Doudoroff pathway is an alternative way to break down glucose into two pyruvate molecules (Fig. A.3). Because it generates only one ATP and one NADH (Table A.2), it is less efficient than glycolysis. This pathway is almost exclusively found in bacteria (e.g., *Pseudomonas* and *E. coli*, as well as other Gram negatives) and certain archaea. Some obligate aerobes use this pathway because they cannot convert glucose to glyceraldehyde 3-phosphate, a necessary intermediate in glycolysis. The pathway also helps cells metabolize a different category of sugars (aldonic acids) than glycolysis, enhancing the chances for survival by broadening the organism's range of nutrients.

The pentose-phosphate pathway is a complex set of cyclic reactions that provides a mechanism for producing 5-carbon sugars (pentoses) from 6-carbon sugars (hexoses) (Fig. A.4). The resulting pentose sugars can enter pathways that make ribonucleotides and deoxyribonucleotides, as well as aromatic amino acids. Further, this pathway produces NADPH, an important electron donor in anabolic pathways.

TABLE **A.1** Summary of Key Glycolytic Reactants and Products per Glucose (Theoretical Maximum Yield)

Reactant	Product
Glucose ($C_6H_{12}O_6$)	2 Pyruvate ($C_3H_3O_3$)
2 ATP + 4 ADP + 2 P_i	4 ATP + 2 ADP (***Note:*** The *profit* is 2 ATP when we consider the initial 2 ATP investment)
2 NAD^+	2 NADH

TABLE **A.2** Summary of Entner-Doudoroff Reactants and Products per Glucose (Theoretical Maximum Yield)

Reactant	Product
Glucose ($C_6H_{12}O_6$)	2 Pyruvate ($C_3H_3O_3$)
1 ATP + 2 ADP + P_i	1 ADP + 2 ATP (***Note:*** The *profit* is 1 ATP when we consider the initial ATP investment)
1 NAD^+	1 NADH
1 $NADP^+$	1 NADPH

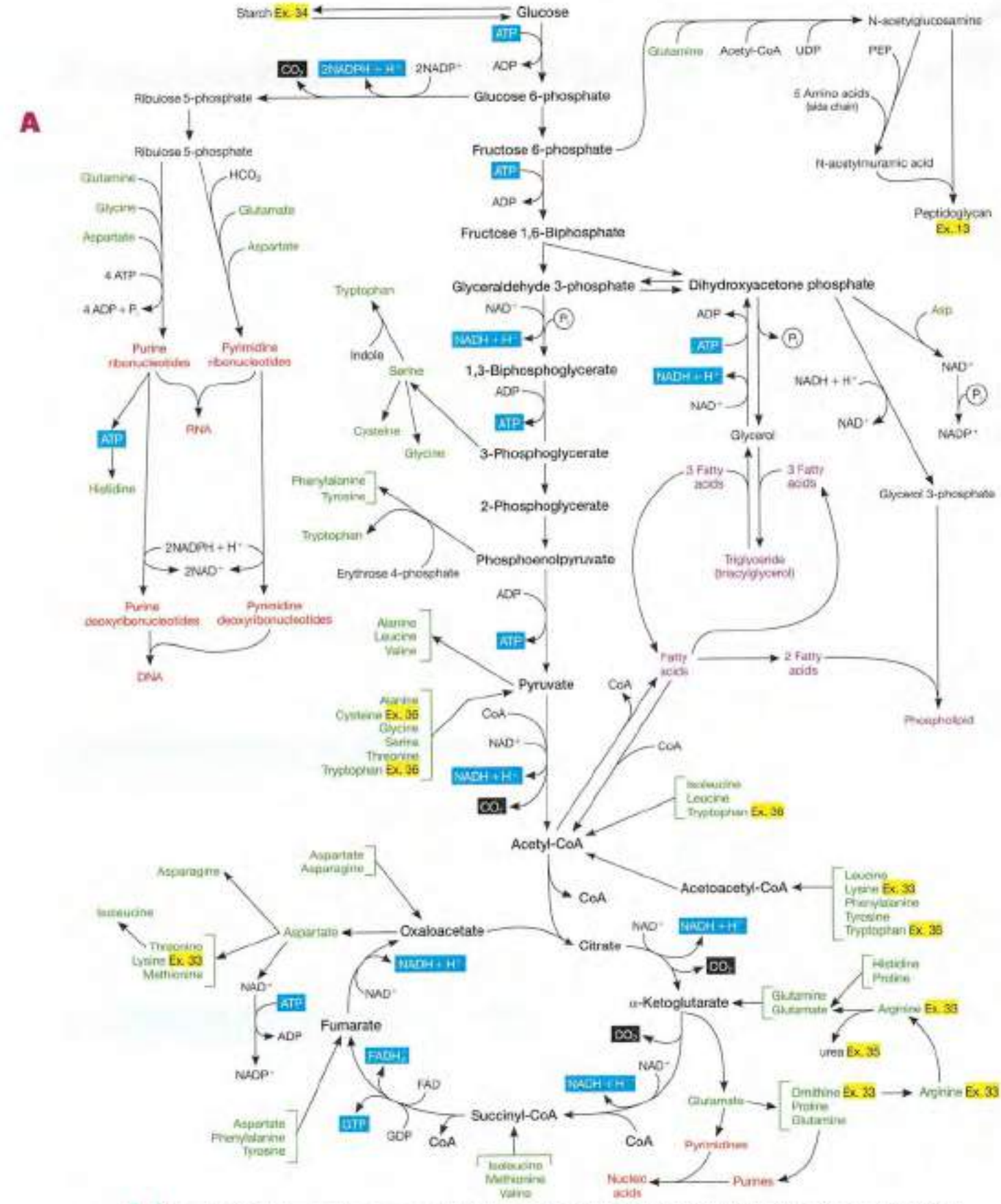


FIGURE **A.1** **Integrated Metabolism** • This diagram shows select bacterial anabolic and catabolic pathways and examples of where lab exercises touch on these metabolic pathways. **Abbreviations: CoA**: coenzyme A; **P_i**: inorganic phosphate; **GTP**: guanosine triphosphate; **GDP**: guanosine diphosphate; **UDP**: uridine diphosphate; **NAD$^+$/NADH**: nicotinamide adenine dinucleotide (oxidized and reduced forms); **NADP$^+$/NADPH**: nicotinamide adenine dinucleotide phosphate (oxidized and reduced forms); **FAD/FADH$_2$**: flavin adenine dinucleotide (oxidized/reduced).

The pentose-phosphate reactants and products are listed in Table A.3. To completely oxidize one hexose to 6 CO_2, a total of six hexoses must enter the cycle as glucose 6-phosphate and follow one of three different routes (notice the symmetry of pathways as drawn). Notice in Figure A.4 that each hexose loses a CO_2 upon entering the cycle, but at the end five hexoses are produced. Thus, the net reaction is one hexose being oxidized to 6 CO_2. Notice also the reactions that transfer 2-carbon and 3-carbon fragments between the 5-carbon intermediates. Transketolase catalyzes the 2-carbon transfer, whereas transaldolase catalyzes the 3-carbon transfer. Alternatively, the 5-carbon intermediates can be redirected into pathways for synthesis of aromatic amino acids and nucleotides (not shown).

The decarboxylation of glucose 6-phospate in the pentose phosphate pathway is not as simple as shown in Figure A.4. The preliminary steps include production of 6-phosphogluconate (an Entner-Doudoroff pathway intermediate), to accommodate the passage of carbon skeletons between the two pathways. The pentose phosphate pathway also intersects with glycolysis at glyceraldehyde 3-phosphate and fructose 6-phosphate.

TABLE **A.3** Summary of Pentose-Phosphate Reactants and Products per Glucose 6-phosphate (Theoretical Maximum Yield) •

Reactant	Product
Glucose 6-phosphate (C_6)	6 CO_2 + 1 P_i
12 $NADP^+$	12 NADPH

Oxidation of Pyruvate: The Citric Acid Cycle and Fermentation

Pyruvate represents a major crossroad in metabolism, and all three pathways discussed so far have the potential to make it, though not in equal quantities. Pyruvate has a variety of possible fates. It can be funneled toward the citric acid cycle, which produces ATP and NADH. Or, in some cases, it can enter fermentation pathways, which generally do not produce ATP.

Organisms that carry out aerobic and/or anaerobic respiration tend to rely on the citric acid cycle as a major metabolic pathway (Fig. A.5). Before the pyruvate enters the citric acid cycle, an intermediate (or gateway) step converts it to acetyl-coenzyme A (acetyl-CoA). Then, acetyl-CoA enters the citric acid cycle through a condensation reaction with oxaloacetate.

For each pyruvate that enters the cycle via the intermediate (or entry) step, the following products are made: 3 CO_2, 4 $NADH^+$, 1 $FADH_2$, and 1 GTP. (Because two pyruvate molecules are made per glucose, these numbers are doubled in Table A.4, which shows the theoretical maximum yield per glucose.) Electron transport chains then oxidize NADH and $FADH_2$, thereby fueling ATP production. Table A.5 shows theoretical maximum ATP yields from the complete oxidation of glucose.

Like glycolysis, many of the citric acid cycle's intermediates are entry points for amino acid, nucleotide, and lipid catabolism, as well as a source of carbon skeletons for synthesis of the same compounds. These pathways are shown in Figure A.1. Single arrows may represent several reactions, and other carbon compounds, not illustrated, may be required to complete a given reaction.

Figure A.6 illustrates some major microbial fermentation pathways (though no single organism performs all of them). Pyruvate (shown in the blue box) is typically the starting point for each. Fermentation end products are shown in red. Fermentation allows some cells living under anaerobic conditions to replenish the supply of oxidized coenzymes (e.g., convert NADH back to NAD^+, shown in blue) generated during glycolysis or other pathways. Some bacteria (aerotolerant anaerobes) exclusively rely on fermentation, even if oxygen is available. Table A.6 summarizes major fermentation pathways and representative organisms that perform each.

Notice that fermentation end products typically fall into three categories: acid, gas, or an organic solvent (an alcohol or a ketone). The specific fermentation performed is useful for bacterial classification.

TABLE **A.4** Summary of Reactants and Products per Glucose for the Intermediate Step and Citric Acid Cycle (Theoretical Maximum Yield) ●

Intermediate (or entry) Step		Citric Acid Cycle	
Reactant	**Product**	**Reactant**	**Product**
2 Pyruvate + 2 Coenzyme A	2 Acetyl-CoA + 2 CO_2	2 Acetyl-CoA	4 CO_2 + 2 Coenzyme A
2 NAD^+	2 NADH	6 NAD^+	6 NADH
		2 GDP + 2 P_i (= 2 ADP + 2 P_i)	2 GTP (= 2 ATP)
		2 FAD	2 $FADH_2$

TABLE **A.5** Prokaryotic Aerobic Cellular Respiration Theoretical Maximum ATP Yields ●

Compound	Number Produced	ATP Value[1]	Total ATP per Glucose
NADH (from glycolysis, intermediate step, and citric acid cycle)	10	3	30
$FADH_2$ (from citric acid cycle)	2	2	4
ATP (from substrate phosphorylation in glycolysis)	4		4

[1] Experimental evidence indicates that the ATP values of 3 per NADH and 2 per $FADH_2$ in the aerobic electron transport chain are probably too high. For mitochondria, values of 2.5 and 1.5, respectively, are closer to reality. However, because multiple electron transport chains have been identified within the domain *Bacteria* (and even within single species!) no single, universal number for ATP yields per NADH and $FADH_2$ can be assigned. So, we fall back on the accepted theoretical maximum of 3 and 2, respectively.

TABLE **A.6** Major Fermentations, Their End Products, and Some Organisms That Perform Them ●

Fermentation	Major End Products	Representative Organisms
Alcoholic fermentation	Ethanol and CO_2	*Saccharomyces cerevisiae*
Homolactic fermentation	Lactate	*Streptococcus* and some *Lactobacillus*
Heterolactic fermentation	Lactate, ethanol, and acetate	*Streptococcus, Leuconostoc,* and *Lactobacillus*
Mixed acid fermentation	Acetate, formate, succinate, CO_2, H_2, and ethanol	*Escherichia, Salmonella, Klebsiella,* and *Shigella*
2,3-butanediol fermentation	2,3-butanediol	*Enterobacter, Serratia,* and *Erwinia*
Butyrate/butanol fermentation	Butanol, butyrate, acetone, and isopropanol	*Clostridium, Butyrivibrio,* and some *Bacillus*
Propionic acid fermentation	Propionate, acetate, and CO_2	*Propionibacterium, Veillonella,* and some *Clostridium*

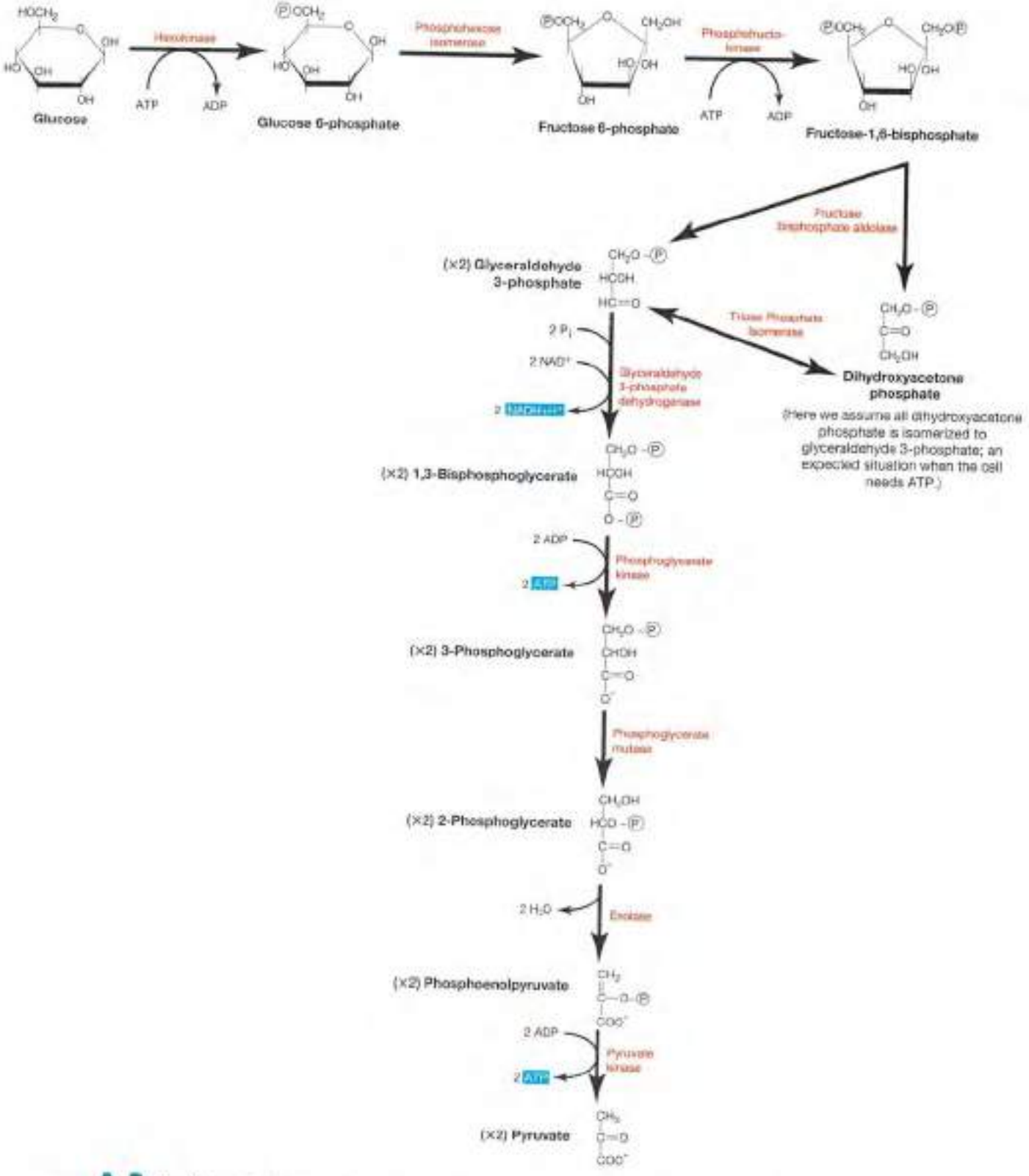


FIGURE **A.2** **Glycolysis** • Glycolytic intermediates are in black; the enzyme names are in red. Reducing power (NADH) and ATP are highlighted in blue. The pathway shown here assumes that all dihydroxyacetone phosphate is isomerized to glyceraldehyde 3-phosphate, the expected situation when a cell needs ATP. Table A.3 summarizes the products and reactants of glycolysis.

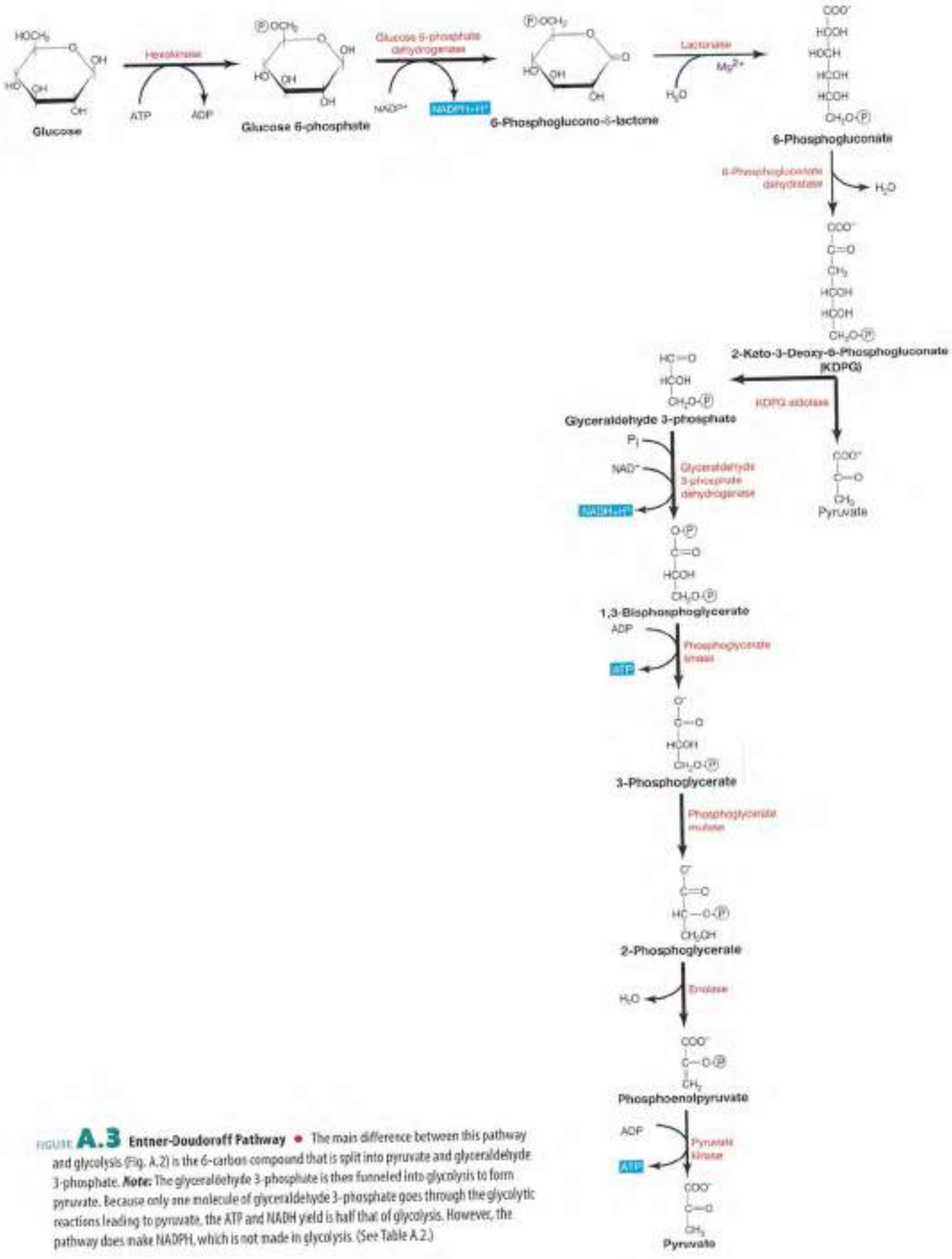


FIGURE **A.3** **Entner-Doudoroff Pathway** • The main difference between this pathway and glycolysis (Fig. A.2) is the 6-carbon compound that is split into pyruvate and glyceraldehyde 3-phosphate. ***Note:*** The glyceraldehyde 3-phosphate is then funneled into glycolysis to form pyruvate. Because only one molecule of glyceraldehyde 3-phosphate goes through the glycolytic reactions leading to pyruvate, the ATP and NADH yield is half that of glycolysis. However, the pathway does make NADPH, which is not made in glycolysis. (See Table A.2.)

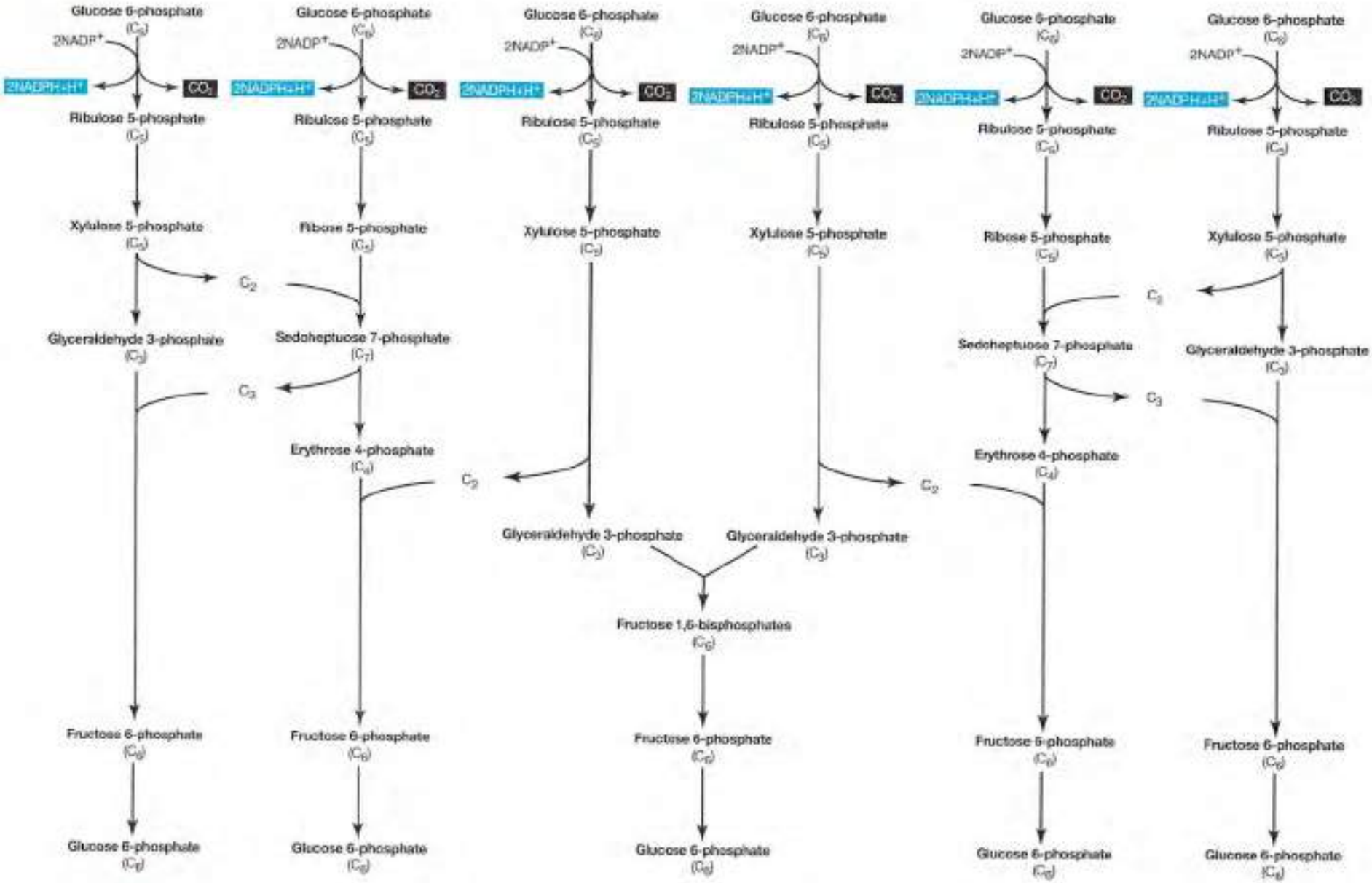


FIGURE **A.4** **Pentose-Phosphate Cycle** • For every six glucose 6-phosphates that enter and complete the cycle, 6 CO_2 and 12 NADPH are produced. Some of the 5-carbon intermediates, however, may be used to build aromatic amino acids and nucleotides. If the cycle is performed as shown, 36 carbons enter as six glucose 6-phosphates ($6 \times C_6 = 36C$). Six CO_2 are immediately lost, leaving a total of 30C to get shuffled around by the remaining reactions to form five glucose 6-phosphates ($5 \times C_6 = 30C$). (See Table A.3.)

A

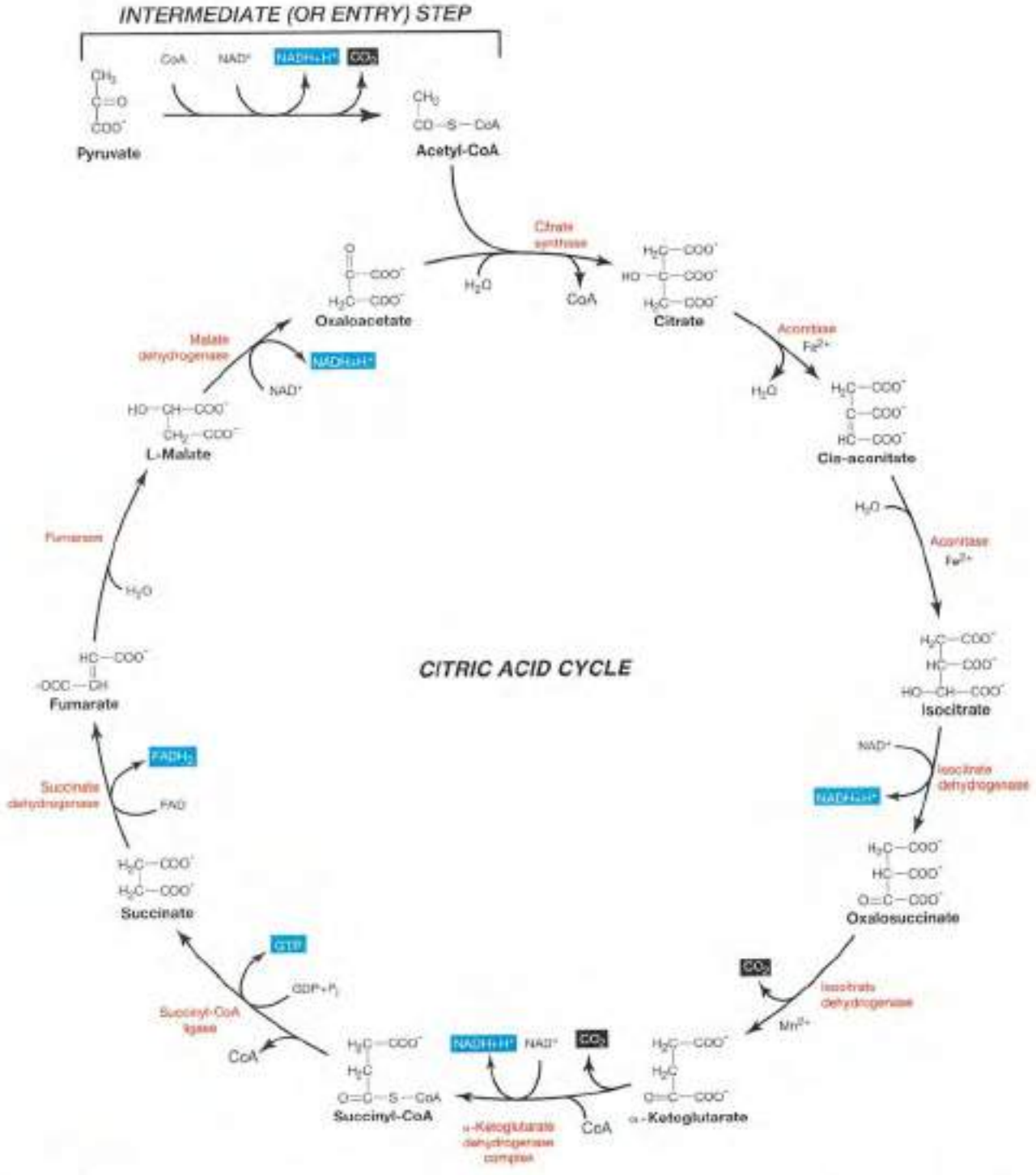
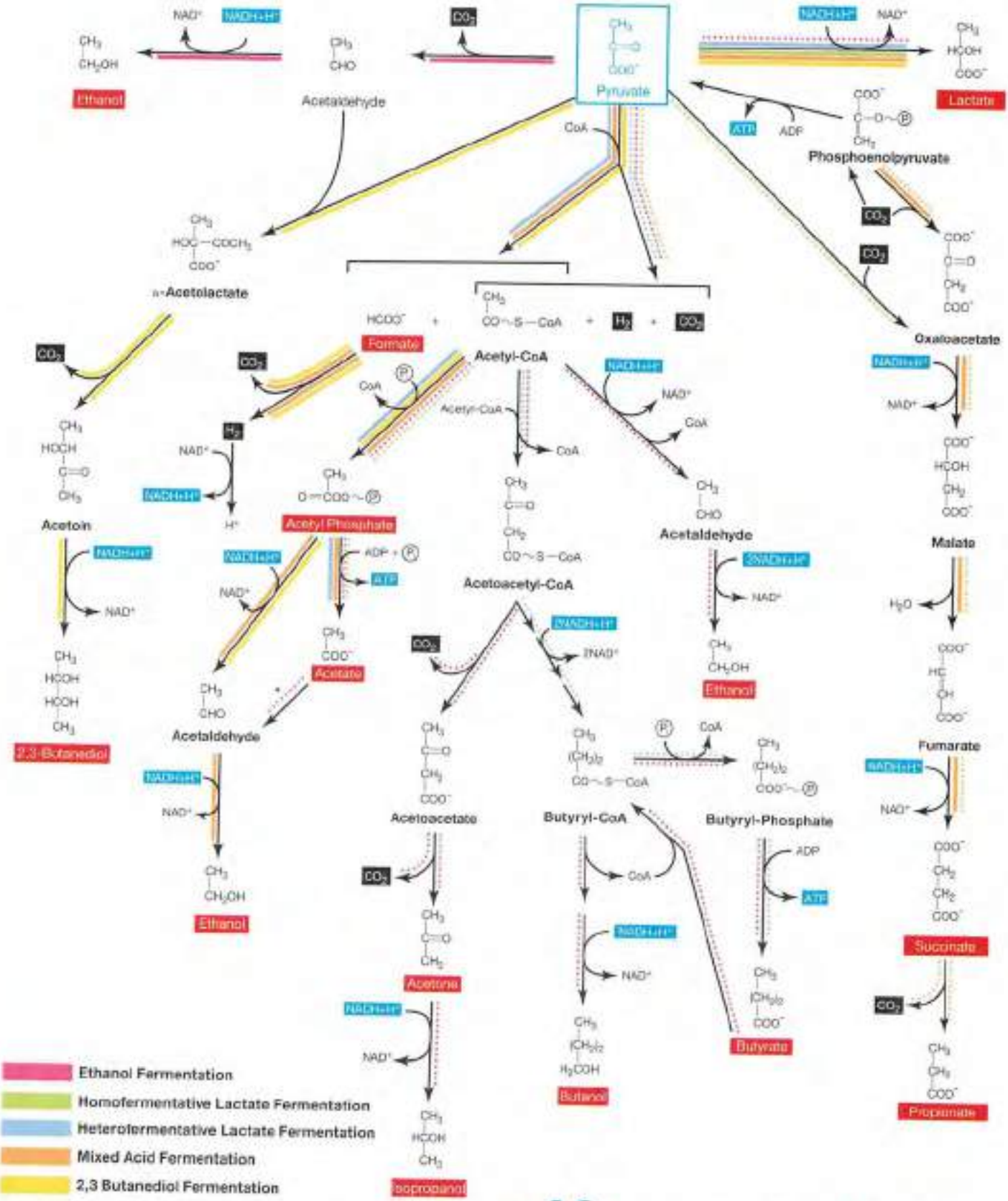


FIGURE **A.5** **Intermediate Step and Citric Acid Cycle** • Intermediates are printed in black; enzymes are in red. Reducing power (in the form of NADH and $FADH_2$) and energy (in the form of GTP) are highlighted in blue. CO_2 produced from the oxidation of carbon is highlighted in black. (See Table A.4.)



A

FIGURE **A.6** **Sampling of Fermentation Pathways** • Note that all pathways start with pyruvate and, at some point, NADH (in blue) is oxidized to NAD^+. The resulting end products are acids, gases, or alcohols. Major fermentation pathways are highlighted by different colors and are summarized in Table A.5.

— Lab Notes —

APPENDIX B
Miscellaneous Transfer Methods

Following are instructions for transfer methods that are performed less routinely than those in Exercises 3 and 4.

Transfers Using a Sterile Cotton Swab

A sterile swab generally is used to obtain a sample from a primary source, either a patient or an environmental site. Occasionally, swabs are used to transfer pure cultures (e.g., Kirby-Bauer test, Ex. 43, pp. 389–395). Sterile swabs may be dry, or they may be in sterile water, depending on the nature of the sample to be taken (wet or dry). In either case, care must be taken not to contaminate the swab by unintentionally touching other surfaces with it. Your instructor may provide specific instructions on collection of samples from sources other than the ones listed here.

Obtaining a Sample from a Patient's Throat with a Swab

1. **Use BSL-2 precautions throughout.** Wear a lab coat, disposable gloves, and eye protection.
2. Use a sterile tongue depressor and swab prepared in sterile water to obtain a sample from the throat. Have the patient open their mouth, and then gently press down on the tongue (Fig. B.1).
3. With the swab in your dominant hand, carefully sample the patient's throat with a swirling motion. Touching other parts of the oral cavity is likely to cause contamination. Also, avoid touching the soft palate or it may initiate a gag reflex!
4. Transfer the sample to an appropriate plated medium as quickly as possible. If plating is to be done at a later time, place the swab in an appropriate sterile container (such as a sterile, capped test tube with sterile water deep enough to immerse the cotton). Store in the lab refrigerator if necessary.
5. Use the swab to begin a quadrant streak (Ex. 4, Fig. 4.3, p. 70). Properly dispose of the swab according to your lab's protocol, and then complete the streaking with your loop. Incinerate the loop or dispose of it in the appropriate autoclave container.
6. Label the plate with your name, the sample source, date, and medium.

FIGURE **B.1** **Taking a Throat Sample** • Use a sterile tongue depressor and swab to obtain a sample from a patient's throat. Be careful not to touch other parts of the oral cavity or the sample will get contaminated. (The communities of microorganisms differ in different parts of the oral cavity and throat!) Transfer the sample to a sterile medium as soon as possible.

7. Incubate the plate in an inverted position at the appropriate temperature for the designated time.

Obtaining an Environmental Sample with a Cotton Swab

1. **Use BSL-2 precautions throughout.** Wear a lab coat, disposable gloves, and eye protection.
2. Use a sterile swab prepared in sterile water to obtain a sample from an environmental source.
3. Rotate the swab to collect from the area to be sampled (Fig. B.2).
4. Transfer the sample to an appropriate plated medium as quickly as possible. If plating is to be done at a later time, place the swab in an appropriate sterile container (such as a sterile, capped test tube with sterile water deep enough to immerse the cotton). Store in the lab refrigerator if necessary.
5. Depending on the anticipated cell density, use the swab to perform a zigzag streak pattern (Ex. 4, Fig. 4.6, p. 72) or to begin a quadrant streak with a disposable loop (Ex. 4, Fig. 4.3, p. 70). Properly dispose of the swab and loop (if used) in the appropriate autoclave container.

FIGURE **B.2** **Taking an Environmental Sample** • Use a spinning motion of a sterile swab to sample inanimate objects in the environment. The swab may be placed in a sterile test tube until it is convenient to transfer the sample to a growth medium. Notice the tube's cap being held in the pinky of the swab hand.

6 Label the plate with your name, the sample source, date, and medium.

7 Incubate the plate in an inverted position at the appropriate temperature for the designated time.

Spot Inoculation of an Agar Plate

Sometimes an agar plate may be used to grow several different specimens at once. This is a typical practice with plated *differential* media (i.e., media designed to differentiate organisms based on growth characteristics).

Prior to beginning the transfer, divide the plate into as many as four sectors using a marking pen. (Some plates already have marks on the base for this purpose.) Then each may be inoculated with a different organism.

Inoculation involves touching the loop to the agar surface once so growth is restricted to a single spot, hence the name "spot inoculation."

1 Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

2 Label the plate's base with your name, date, medium, and organism(s) inoculated. Write these toward the periphery, not across the center, which makes results difficult to read.

3 With a marking pen, divide the base of the plate into the desired number of sectors. Figure B.3 shows a plate divided into four sectors.

4 Aseptically get growth from the source culture on the inoculating loop.

5 Lift the lid of the sterile agar plate and use it as a shield to prevent airborne contamination.

6 Touch the agar surface toward the periphery of the sector (Fig. B.3).

7 Remove the loop and replace the lid.

8 Incinerate your loop as before. It is especially important to flame it from base to tip now because the loop has bacteria on it.

9 Repeat Steps 4 through 8 for the remaining cultures.

10 Incubate the plate in an inverted position for the assigned time at the appropriate temperature.

11 Save or dispose of the original cultures as directed by your instructor.

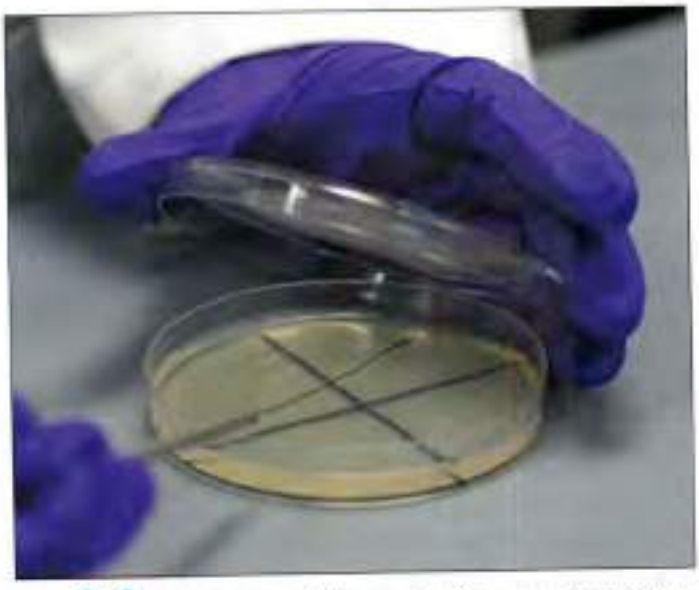


FIGURE **B.3** **Spot Inoculation of a Plate** • Each of four sectors is spot inoculated with a different organism by touching the loop to the surface and making a mark about 1 cm in length. Generally, spot inoculations are done toward the edge (rather than the crowded middle) to prevent overlapping growth and/or test results.

APPENDIX C

Using a Glass Pipette

Glass pipettes are used to transfer a known volume of liquid diluent, media, or culture. Originally pipettes were filled by sucking on them like a drinking straw, but mouth pipetting is dangerous and has been replaced by mechanical pipettes. Three examples are shown in Figure C.1, each with its own method of operation. Your instructor will show you how to properly use the type of pipette available in your lab.

To use a pipette correctly, you must be able to correctly read the calibration. Examine Figure C.2. The numbers at the top indicate the pipette's *total volume* and its *smallest calibrated increments*. This is a 10.0 mL pipette divided into 0.1 mL increments.

When reading volumes, use the base of the meniscus (Fig. C.3). The volume in the center pipette is read as exactly 3.0 mL because the meniscus is resting on the line. The right pipette is read as 2.9 mL and the left pipette is read as 3.1 mL (0 is always at the top of the pipette). Although the difference in volume between these three pipettes may seem negligible (1 part in 70[1] is about a 1.4% error), it introduces avoidable error into your work.

Two pipette styles are used in microbiology (Fig. C.4): the serological pipette and the Mohr pipette. A serological pipette is calibrated to deliver (TD) its volume by completely draining it and blowing out the last drop. The tip of a Mohr pipette is not graduated, so fluid flow must be stopped at a calibration line. Stopping the fluid beyond the last line on a Mohr pipette results in an unknown volume being dispensed. In either case, volumes are read at the bottom of the fluid meniscus.

[1] This number presumes the pipette shown has a total volume of 10 mL. If the meniscus is on the 3, the pipette will dispense 7 mL. If emptied completely, an error of 0.1 mL above (delivering 7.1 mL) or below the 3 (delivering 6.9 mL) would represent 0.1 mL in 7 mL, or 1 part in 70, a 1.4% error that is avoidable.

CAUTION!

It's important if you are pipetting a bacterial culture to work over a disinfectant-soaked towel in case any culture drips from the pipette before disposing of it in the autoclave container. This is especially important if transferring a BSL-2 organism. Clean up any spills with additional disinfectant. Also, it is recommended that you use a Mohr pipette to transfer BSL-2 organisms. These are not "blown-out," so aerosol production is reduced.

FIGURE **C.1** **Mechanical Pipettes** • Three examples of mechanical pipettes are shown here, each with its own method of operation. Your instructor will show you how to properly use the style of pipette available in your lab. From left to right: A semi-automated piette, a pipette bulb, and a plastic pump.

FIGURE **C.2** **Pipette Calibration** • Prior to using a pipette, read its calibration. The numbers indicate the pipette's total volume and its smallest calibrated increments. This is a 10.0 mL pipette divided into 1/10 (0.1) mL increments.

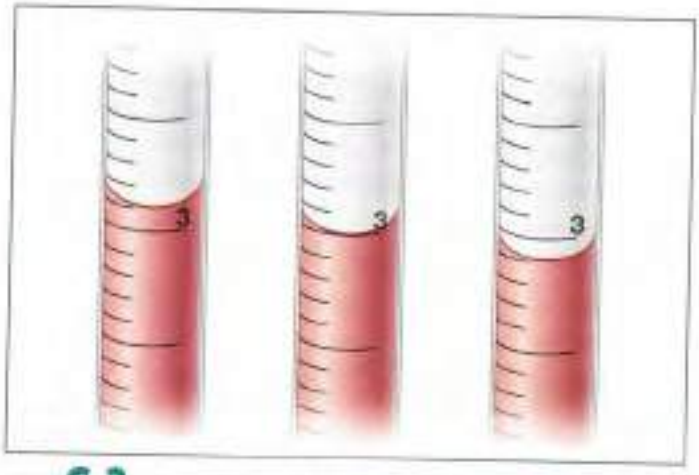


FIGURE **C.3** **Read the Base of the Meniscus** • When reading volumes, use the base of the meniscus. The volume in the center pipette is read at exactly 3.0 mL because the meniscus is resting on the line. The right pipette is read as 2.9 mL and the left pipette is read as 3.1 mL (0 is always at the top of the pipette). ***Note:*** The number read usually isn't the volume delivered. If these are all 10.0 mL pipettes, then completely emptying the center one would deliver 10.0 mL – 3.0 mL = 7.0 mL. The pipette on the right would deliver 7.1 mL and the one on the left 6.9 mL.

FIGURE C.4 Two Types of Pipettes • Two pipette styles—the serological pipette (left) and the Mohr pipette (right)—are used in microbiology. A serological pipette is calibrated to deliver (TD) its volume by completely draining it and blowing out the last drop. The tip of a Mohr pipette is not graduated, so fluid flow must be stopped at a calibration line. Stopping the fluid beyond the last line on a Mohr pipette results in an unknown volume being dispensed.

C

Filling a Glass Pipette

1. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.
2. Bacteria should be suspended in the broth with a vortex mixer or by agitating with your fingers (Ex. 3, Fig. 3.6, p. 54). Be careful not to splash the broth into the cap or lose control of the tube. If transferring a sterile liquid, mixing is not necessary.
3. Pipettes are sterilized in metal canisters, individually in sleeves, or as multiples in packages (if disposable). They typically are stored in groups of a single size (Fig. C.5). *Be sure you know what volume your pipette will deliver.* Set the canister at the table edge and remove its lid. (Canisters—even cylindrical ones—should not be stored in an upright position, because they may fall over and break the pipettes or become contaminated.) If using pipettes in a package, open the end *opposite the tips.* Grasp *one pipette only* and remove it.
4. Carefully insert the pipette into the mechanical pipette (Fig. C.6). Grasp the pipette near the end with your fingertips. This gives you more control and reduces the chance that you will break the pipette and cut your hand or jam the broken pipette into your palm. **Notice how the microbiologist is holding it in Figure C.6.** *Do not touch* any part of the pipette that will contact the specimen or the medium or you risk introducing a contaminant. Also, do not lay the pipette on the tabletop while you continue.
5. While keeping your pipette hand still, bring the culture tube toward it. Use your little finger to remove and hold its cap.
6. Flame the open end of the tube by passing it through a Bunsen burner flame two or three times.
7. Hold the tube at an angle to prevent contamination from above.
8. Insert the pipette and withdraw the appropriate volume (Fig. C.7). Bring the pipette to a vertical position briefly to read the meniscus accurately. (**Remember:** The volumes in the pipette are correct only if the meniscus of the fluid inside is resting *on* the line, not above or below it.) Then carefully remove the pipette from the tube.

FIGURE C.5 Getting the Sterile Pipette • Pipettes of the same size are autoclaved in canisters, which then are opened and placed flat on the table for stability. Pipettes are removed as needed by only touching the end opposite the tip.

FIGURE C.6 Assembling the Pipette • Carefully insert the pipette into a mechanical pipette. Notice that the pipette is held near the end with the fingertips. For safety, the hand is out of the way in case the pipette breaks.

FIGURE **C.7** **Filling the Pipette** • Carefully draw the fluid into the pipette. Briefly bring it to a vertical position and read the volume. Notice how the tube's cap is being held by the pinky finger of the pipette hand.

9 Flame the tube lip as before. Keep your pipette hand still.

10 Keeping the pipette hand still (remember, it may contain fluid with microbes in it), move the tube to replace its cap.

11 What you do next depends on the medium to which you are transferring the liquid. Please continue with the appropriate inoculation section.

Inoculation of Broth Tubes with a Pipette

Pipettes are often used to inoculate a known volume of culture into a tube or bottle containing a known volume of diluent during serial dilutions. They also would have been used to deliver the volume of diluent waiting in the tube or bottle.

1 While keeping the pipette hand still, bring the dilution tube/bottle toward it. Use your little finger to remove and hold its cap.

2 Flame the lip of the tube/bottle by quickly passing it through the Bunsen burner flame two or three times. Keep your pipette hand still.

3 Hold the open tube/bottle on an angle to minimize airborne contamination. Keep your pipette hand still.

4 Insert the pipette tip and gently dispense the correct volume of inoculum. Aerosols can be produced if you dispense too quickly.

5 Withdraw the tube/bottle from over the pipette. If using a serological pipette, touch its tip to the glass to remove any excess broth before completely removing it.

6 Completely remove the pipette, but avoid waving it around. This can create aerosols.

7 Flame the tube/bottle lip as before. Keep your pipette hand still.

8 Keeping the pipette hand still, move the tube/bottle to replace its cap.

9 The pipette may be contaminated with microbes and must be disposed of correctly. Each lab has its own specific procedures, and your instructor will advise you what to do. Glass pipettes typically are placed in a pipette disposal receptacle containing a small amount of disinfectant until they are autoclaved and reused (Fig. C.8). Disposable pipettes must be placed in an appropriate biohazard container. In either case, be careful when removing the pipette from the mechanical pipette. There is danger of culture dripping from the pipette or of breaking the glass or plastic.

FIGURE **C.8** **Dispose of the Pipette** • The pipette must be disposed of correctly, especially if it is contaminated with microbes. Each lab has its own specific procedures, and your instructor will advise you what to do. Shown here is a jar used for reusable glass pipettes. Disposable pipettes must be placed in an appropriate biohazard container. In either case, be careful when removing the pipette from the mechanical pipette. There is danger of culture dripping from the pipette or of breaking the glass or plastic.

Inoculation of Agar Plates with a Pipette

Pipettes also can be used to dispense a known volume of inoculum to an agar plate.

1. Lift the lid of the plate and use it as a shield to protect it from airborne contamination. It also will help in containing any aerosols you inadvertently produce delivering the inoculum.
2. Hold the pipette over the agar and dispense the correct volume (often 0.1 mL) onto the agar surface between the middle and one edge of the dish (Fig. C.9). From this point, the remaining steps should be completed within about 15 seconds to prevent the inoculum from soaking into the agar.
3. The pipette is contaminated with microbes and must be disposed of correctly. Each lab has its own specific procedures, and your instructor will advise you what to do. Glass pipettes typically are placed in a pipette disposal receptacle containing a small amount of disinfectant until they are autoclaved and reused (Fig. C.8). Disposable pipettes must be placed in an appropriate biohazard container. In either case, be careful when removing the pipette from the mechanical pipette. There is danger of culture dripping from the pipette or of breaking the glass or plastic.
4. Continue with the spread plate technique (Ex. 5, pp. 81–84).

FIGURE **C.9** **Dispensing to an Agar Plate** • Open the lid and dispense the inoculum onto the surface of the agar between the middle and edge. This provides open agar on which to cool the spreading rod. Notice how the plate's lid is held to prevent aerial contamination and to minimize aerosols. The plate may be placed on a disinfectant-soaked towel in case the pipette drips (not shown).

APPENDIX D
Using a Micropipette

Modern molecular biology procedures often involve transferring extremely small volumes of liquid with great precision and accuracy. This has led to the development of micropipettes (sometimes called a digital adjustable pipette) (Fig. D.1) that can be set to dispense microliter volumes through milliliter volumes (recall that 1 mL equals 1,000 µL). Common micropipette calibrations include volumes of 1 to 10 µL, 10 to 100 µL, 100 to 1,000 µL, or 1,000 to 5,000 µL.

Filling a Micropipette

Many manufacturers make micropipettes, but they all work basically the same way. The following instructions are for Eppendorf Series 2100 models. ***Caution!*** **Use BSL-2 precautions, if appropriate.** Before you begin, label the destination tube(s) or plate(s) with your name, the date, source of inoculum, and medium.

1. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.
2. Growth may be suspended in the broth with a vortex mixer or by agitating with your fingers (Ex. 3, Fig. 3.6, p. 54). Be careful not to splash the broth into the cap or lose control of the tube. If transferring a sterile liquid, mixing is not necessary.
3. Determine which micropipette should be used to dispense the desired volume.
4. Turn the setting ring to set the desired volume (Fig. D.2). Do not turn the dial past either extreme of the pipette's volume range. Doing so will damage it.
5. Hold the micropipette in your dominant hand.
6. Open the case of appropriate pipette tips for your pipette (these are often color-coded and match the pipette) and push the pipette into a sterile tip (Fig. D.3). Close the case. Do not touch the pipette tip with your hands or leave the case open. **Never use a micropipette without a tip.**
7. Remove the cap from the culture tube with the little finger of your dominant hand, and flame the tube. If transferring from a microtube, simply open the cap, but don't flame it!
8. Press down the control button with your thumb to the first stop. This is the measuring stroke.
9. Insert the tip into the broth approximately 3 mm while holding it vertically (Fig. D.4). Make sure you put the tip in deep enough—but not too deep—so it doesn't come out as the liquid level drops while filling.

FIGURE **D.2** **Setting the Volume** • Rotate the adjustment knob (setting ring) to set the volume on a micropipette. This pipette has been set at 90.0 µL (the line between the last two zeroes is a decimal point). Never rotate the adjustment knob beyond the volume limits of the pipette as it may become damaged.

FIGURE **D.1** **Micropipette** • This is a micropipette that dispenses volumes between 10 µL and 100 µL. Also, always use a micropipette with the appropriate tip. As shown here, tips and pipettes are often color-coded. If the tips have been sterilized, always close the tip case when not picking up a tip.

FIGURE **D.3** **Micropipette Tips** • Micropipettes must be fitted with a (sterile) tip of appropriate size (often color-coded to match the pipette). Open the case and press the pipette into a tip, then close the case to maintain sterility. Do not touch the pipette tip.

D

FIGURE **D.4** **Filling the Micropipette** • Depress the control button with your thumb to the first stop (measuring stroke). Holding the tube and pipette in a vertical position, insert the tip deep enough into the fluid (about 3 mm, see inset) that the tip won't be exposed as the liquid level drops during filling. Slowly release pressure with your thumb to fill the pipette.

10 *Slowly* release pressure with your thumb to draw fluid into the tip. Be careful not to pull any air into the tip.

11 Remove the pipette and hold it still as you flame the tube as before or close the microtube's cap (without flaming).

12 Keeping the pipette hand still, move the tube to replace its cap.

13 What you do next depends on the medium to which you are transferring the growth. Please continue with the appropriate inoculation section.

Using a Micropipette to Inoculate Broth Tubes

Micropipettes are often used to inoculate a known volume of culture into a tube or bottle containing a known volume of diluent during serial dilutions. They also would have been used to deliver the volume of diluent waiting in the tube or bottle.

1 Remove the cap of the sterile medium with the little finger of your pipette hand, and hold it there. If using a microtube, simply open the cap and continue with Step 3.

2 Flame the tube by quickly passing it through the Bunsen burner flame a couple of times. Keep your pipette hand still.

3 Insert the pipette tip into the tube. Hold it at an angle against the inside of the tube (Fig. D.5).

4 Depress the control button slowly with your thumb to the first stop and pause when no more liquid is dispensed. Then continue pressing gently to the second stop to deliver the remaining volume. This is the blow-out stroke, but don't do it so forcefully that you splash liquid.

5 While keeping pressure on the control button, carefully remove the pipette from the tube by sliding it along the inside of the tube. Once it is out, slowly release pressure on the control button.

6 Keeping your pipette hand still, flame the tube lip as before. If using a microtube, close the cap and continue with Step 8.

7 Keeping the pipette hand still, move the tube to replace its cap.

8 The pipette tip is contaminated with microbes, and must be disposed of correctly. Use the ejector button to remove the tip into an appropriate

biohazard container (Fig. D.6). Each lab has its own specific procedures and your instructor will advise you what to do.

FIGURE **D.5** **Dispensing to a Liquid** • Hold the pipette on a slight angle with the tip immersed in the liquid and held against the glass of the test tube (or plastic of a microtube). Press the control button gently to the first stop (don't shoot out the liquid!). When no more fluid comes out, continue pressing to the second stop to completely empty the pipette. Remove the pipette along the glass (plastic), then slowly release pressure on the control button and replace the cap on the test tube. Eject the tip into a biohazard container if transferring a culture (Fig. D.6).

Using a Micropipette to Inoculate Agar Plates

1. Lift the lid of the plate and use it as a shield to protect from airborne contamination as well as limiting the spread of any aerosols as you deliver the inoculum.
2. Place the pipette tip over, but not touching, the agar surface. Hold the pipette in a vertical position between the middle and one edge of the plate (Fig. D.7).
3. Depress the control button slowly with your thumb to the first stop, and pause when no more liquid is dispensed. Then continue gently pressing to the second stop to deliver the appropriate volume. This is the blow-out stroke. From this point, the remaining steps should be completed within about 15 seconds to prevent the inoculum from soaking into the agar.
4. Because the pipette tip is contaminated with microbes, you must dispose of it correctly. Use the ejector button to remove the tip into an appropriate biohazard container (Fig. D.6). Each lab has its own specific procedures, and your instructor will advise you what to do.
5. Continue with the spread plate technique (Ex. 5, pp. 81–84).

FIGURE **D.6** **Ejecting the Tip** • Use the tip ejector button to eject the tip into an appropriate biohazard container. Be sure the tip goes into the container and doesn't hit an edge and bounce back. (If this happens, treat it as a spill and wipe down all areas the tip contacted with disinfectant. Manually put the tip in the biohazard container while wearing gloves, then properly remove and dispose of them.) Your instructor will advise you as to the proper procedure in your laboratory. If transferring nonbiohazardous liquids (e.g., sterile water or saline), tips can be ejected into a cup or other suitable container for disposal.

FIGURE **D.7** **Dispensing to a Plate** • Notice how the lid is held as a shield to prevent aerial contamination of the plate and to contain aerosols. Hold the pipette in a vertical position and press the control button to the first stop. When no more fluid is released, continue pressing to the second stop. If transferring a BSL-2 organism, try to deliver the inoculum as far under the lid as is practical. Then, slowly release pressure on the control button and eject the tip into a proper biohazard container (Fig. D.6). Notice the work area is covered with a disinfectant-soaked towel. This is always a good idea in case of drips, but it is a necessity when transferring a BSL-2 organism.

D

Modifications for Transferring BSL-2 Microorganisms

Several modifications to the instructions above should be made to minimize aerosol production when using a micropipette. They may also be employed for other transfers, but are especially important when transferring BSL-2 organisms.

In all cases, transfers should be made over a disinfectant-soaked towel in case inoculum leaks from the pipette tip. Put more disinfectant on any spills. Also, keep the culture and destination media as close together as is safe in order to minimize hand movements.

Following are suggested modifications for transferring BSL-2 organisms with a micropipette.

- The pipette tip containing inoculum may be inserted into the broth. Then, gently deliver the inoculum into the broth. Thus, the "blow-out" stroke (pressing to the second stop) occurs within the broth and does not produce aerosols. However, if you use too much force you can cause bubbling of the broth with concurrent aerosol production, so be sure to *gently* dispense the inoculum. Also be sure to keep pressure on the control button as you withdraw the pipette from the broth or you risk changing an intended concentration as you pull back some broth. Use the ejector button to dispose of the tip according to your lab's conventions with a minimum amount of pipette movement.
- If you are delivering the inoculum to a plate, keep the lid down low and *gently* dispense as far under the lid as possible while still holding the pipette more or less vertically. This will help to contain aerosols. Use the ejector button to dispose of the tip according to your lab's conventions with a minimum amount of pipette movement.
- Another method involves using the pipette "in reverse." Instead of pressing to the first stop to fill, filling can be done by pressing to the *second* stop. Then, instead of dispensing by pressing to the second stop, delivery of the inoculum is done by pressing to the *first* stop to avoid the inevitable spraying that occurs when completely emptying the pipette tip. Use the ejector button to dispose of the tip and the remaining inoculum within it according to your lab's conventions with a minimum amount of pipette movement.

Reference

Brinkmann Instruments, *Eppendorf Series 2100 Pipette Instruction Manual*. Westbury, New York.

APPENDIX E

Alternative Procedure for Exercise 10: Standard Plate Count

What follows is an alternative procedure for Exercise 10, using traditional glass pipettes and mL volumes versus micropipettes and microliter (μL) volumes.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Sterile 0.1 mL, 1.0 mL and 10.0 mL pipettes
- ❑ Mechanical pipette
- ❑ Sterile dilution tubes with caps (5)
- ❑ Flask of 100 mL sterile normal saline or sterile water
- ❑ Nutrient agar (NA) plates (8)
- ❑ Jar (and its lid) containing ethanol, cotton in the bottom, and a bent glass rod
- ❑ Hand-tally counter
- ❑ Colony counter (optional)
- ❑ 24-hour broth culture of *Escherichia coli* (this culture will have between 2×10^7 and 2×10^{10} CFU/mL)

PROCEDURE

Wear a lab coat, gloves, and eye protection when performing this procedure. Refer to the procedural diagram in Figure E.1 as needed.

1. **Label Your Plates and Tubes:** Obtain eight plates, organize them into four pairs, and label them A_1, A_2, B_1, B_2, etc. Obtain five dilution tubes, and label them 1–5, respectively. Make sure they remain covered until needed.
2. **Add Diluent (Water or Saline) to Each Tube:** Aseptically add 9.9 mL sterile water/saline to Dilution Tubes 1 and 2. Cover when finished. Aseptically add 9.0 mL sterile water/saline to Dilution Tubes 3, 4, and 5. Cover when finished.
3. **Make a 10^{-2} Dilution:** Carefully, but thoroughly, mix the broth culture, and aseptically transfer 0.1 mL to Dilution Tube 1. Mix well.
4. **Make a 10^{-4} Dilution:** Aseptically transfer 0.1 mL from Dilution Tube 1 to Dilution Tube 2; mix well.
5. **Make a 10^{-5} Dilution:** Aseptically transfer 1.0 mL from Dilution Tube 2 to Dilution Tube 3; mix well.
6. **Make a 10^{-6} Dilution:** Aseptically transfer 1.0 mL from Dilution Tube 3 to Dilution Tube 4; mix well.
7. **Make a 10^{-7} Dilution:** Aseptically transfer 1.0 mL from Dilution Tube 4 to Dilution Tube 5; mix well.
8. **Inoculate Your Plates:** Aseptically transfer 0.1 mL from Dilution Tube 2 to Plate A_1. Using the spread plate technique (Ex. 5, pp. 81–84), disperse the sample evenly over the entire surface of the agar. Repeat the procedure with Plate A_2, and label both plates "10^{-5} mL original sample," or simply "10^{-5}." Following the same procedure, transfer 0.1 mL volumes from Dilution Tubes 3, 4, and 5 to Plates B, C, and D, respectively. Label the plates accordingly. Allow the inocula to soak into the agar for a few minutes before continuing.
9. **Incubate the Plates:** Invert the plates and incubate at 35°C for 24 to 48 hours.
10. **Dispose of Waste:** Save or dispose of the original *E. coli* culture as directed by your instructor. Tightly cap the dilution tubes, and dispose of them in the appropriate autoclave container.

→ Next Lab Session

1. **Examine Your Samples:** After incubation, examine the plates and determine the countable pair—plates with 30 to 300 colonies. Only one pair of plates should be countable. The remainder should be "too few to count" (TFTC) or "too numerous to count" (TNTC).
2. **Record Your Result:** Count the colonies on both of the "countable plates" and then calculate the average number of colonies. Various methods are available for counting. A simple way is to use a hand-tally counter and a felt-tip marker. Mark each colony on the plastic base and simultaneously click the counter.[1] Record the tallies in the appropriate table of the data sheet, page 125. *Note:* You may have more than one pair of countable plates. For the practice, count all plates that have between

[1] Other, more sophisticated counting methods are available. One uses an electronic pen that, when touched to the plastic petri plate below a colony, records a tally. There are also software systems that capture an image of the plate and then colonies are counted by the computer.

30–300 colonies, and then identify the pair in which you have the most confidence. If no plates are in the 30–300 colony range, count the pair that is closest.

3 **Calculate Original Cell Density (OCD):** Using the formula provided on the data sheet, calculate the sample's OCD and record it in the space provided.

4 **Dispose of Your Plates:** Dispose of the plates in an appropriate autoclave container when finished.

Mix, then 0.1 mL
Mix, then 0.1 mL
Mix, then 1.0 mL
Mix, then 1.0 mL
Mix, then 1.0 mL
Mix, then 0.1 mL
Mix, then 0.1 mL
Mix, then 0.1 mL
Mix, then 0.1 mL
Original sample
Tube 1 9.9 mL diluent 10⁻² dilution
Tube 2 9.9 mL diluent 10⁻⁴ dilution
Tube 3 9.0 mL diluent 10⁻⁵ dilution
Tube 4 9.0 mL diluent 10⁻⁶ dilution
Tube 5 9.0 mL diluent 10⁻⁷ dilution
Plate A
10⁻⁶ mL original sample volume
Plate B
10⁻⁶ mL original sample volume
Plate C
10⁻⁷ mL original sample volume
Plate D
10⁻⁸ mL original sample volume

FIGURE **E.1** **Serial Dilution Procedural Diagram** • This is an illustration of the dilution scheme outlined in the procedure. The dilution assigned to each tube (written below the tube) represents the proportion of original sample inside that tube. For example, if the dilution is 10^{-4}, the proportion of original sample inside the tube would be 1/10,000th of the total volume inside. When 0.1 mL of that solution is transferred to a plate, the volume of sample in the plate is $0.1 \text{ mL} \times 10^{-4} = 10^{-5}$ mL.

APPENDIX F

Morphological Features and Staining Results for Select Bacteria

These results are typical for the species listed. Specific strains may vary because of their genetics, their age, or the environment in which they are grown. Always record your observations in case strain variability leads to misidentification.

Organism	Gram Stain	Cell Morphology	Cell Arrangement[1]	Acid-Fast Stain	Motility (wet mount)[2]	Capsule[3]	Spore Stain
Bacillus subtilis	+	rod	usually single cells	–	+	Varies	+
Corynebacterium xerosis	+	irregular rods	single cells or multiples in angular or palisade arrangement	–	–	–	–
Lactococcus lactis	+	ovoid cocci (appearing stretched in the chain or pair)	chains and some pairs	–	–	+	–
Micrococcus luteus	+	cocci	tetrads (some singles and pairs)	–	–	–	–
Mycobacterium phlei (BSL-2) or *Mycobacterium smegmatis* (BSL-2)	weak +	thin rods	single cells or branched; sometimes in dense clusters	+	–	–	–
Neisseria sicca	–	cocci	pairs and/or tetrads with adjacent sides flattened	–	–	+	–
Pseudomonas putida	–	straight to slightly curved rods	single cells	–	+	–	–
Rhizobium leguminosarum	–	short rods	usually single rods	–	–	+	–
Rhodospirillum rubrum	–	thick spirilla or bent rods	single	–	+	–	–
Shigella flexneri (BSL-2)	–	rods	single	–	–	–	–
Staphylococcus epidermidis	+	cocci	irregular clusters (some singles, pairs, and tetrads)	–	–	–	–

[1] It may be necessary to grow your unknown in broth culture to see the arrangement clearly.

[2] It may be useful to transfer some culture to a small amount of sterile broth (in a microtube, for instance) and incubate the sample for about an hour at 35°C. Then make a wet mount from that culture. See Figure 2.15, page 40, for instructions on making a wet mount. Do not stain your slide.

[3] To be considered a capsule, the white halo needs to be at least the thickness of the cell. If you suspect cells have shrunk, measure them and compare their size and the size of the "capsule" to the cell dimensions as measured in your Gram stain.

— Lab Notes —

APPENDIX G

Gram Stain and Cellular Morphology Flowchart

This sample flowchart may be helpful as you conduct the bacterial unknown lab in Exercise 48.

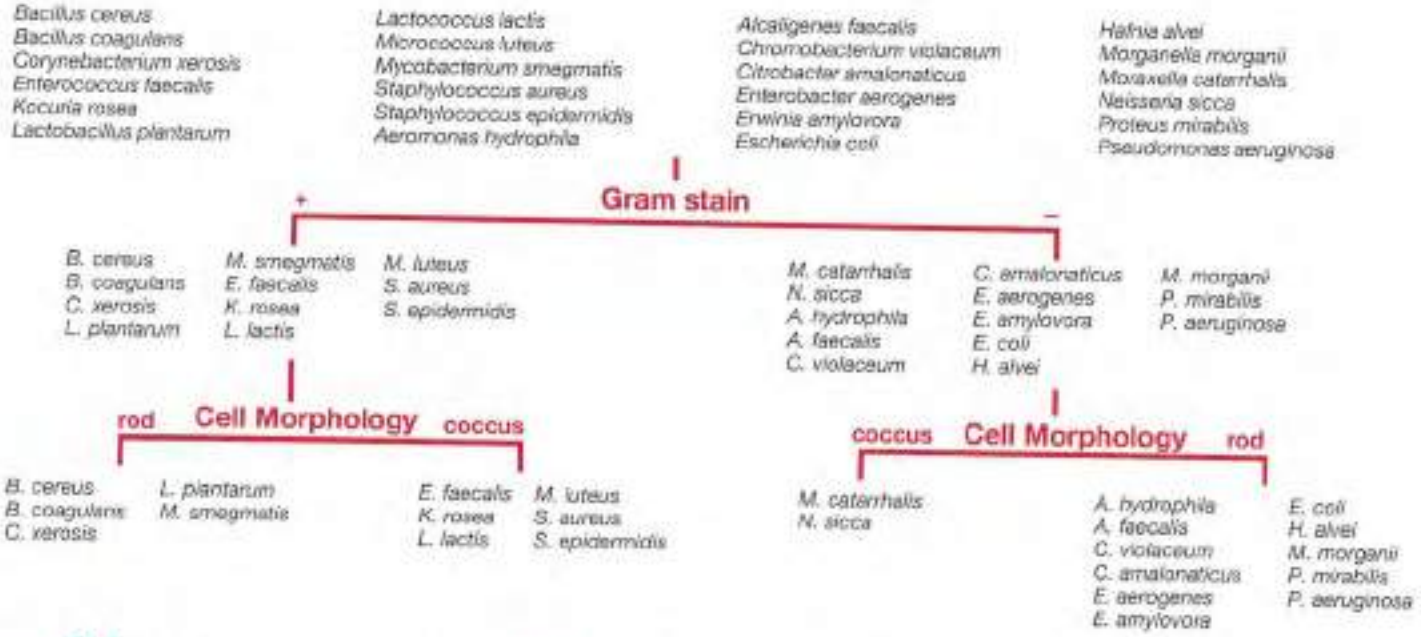


FIGURE **G.1** **Gram Stain and Cellular Morphology Flowchart** • This chart may be useful to you as you work on discovering the identity of your unknown specimen.

G

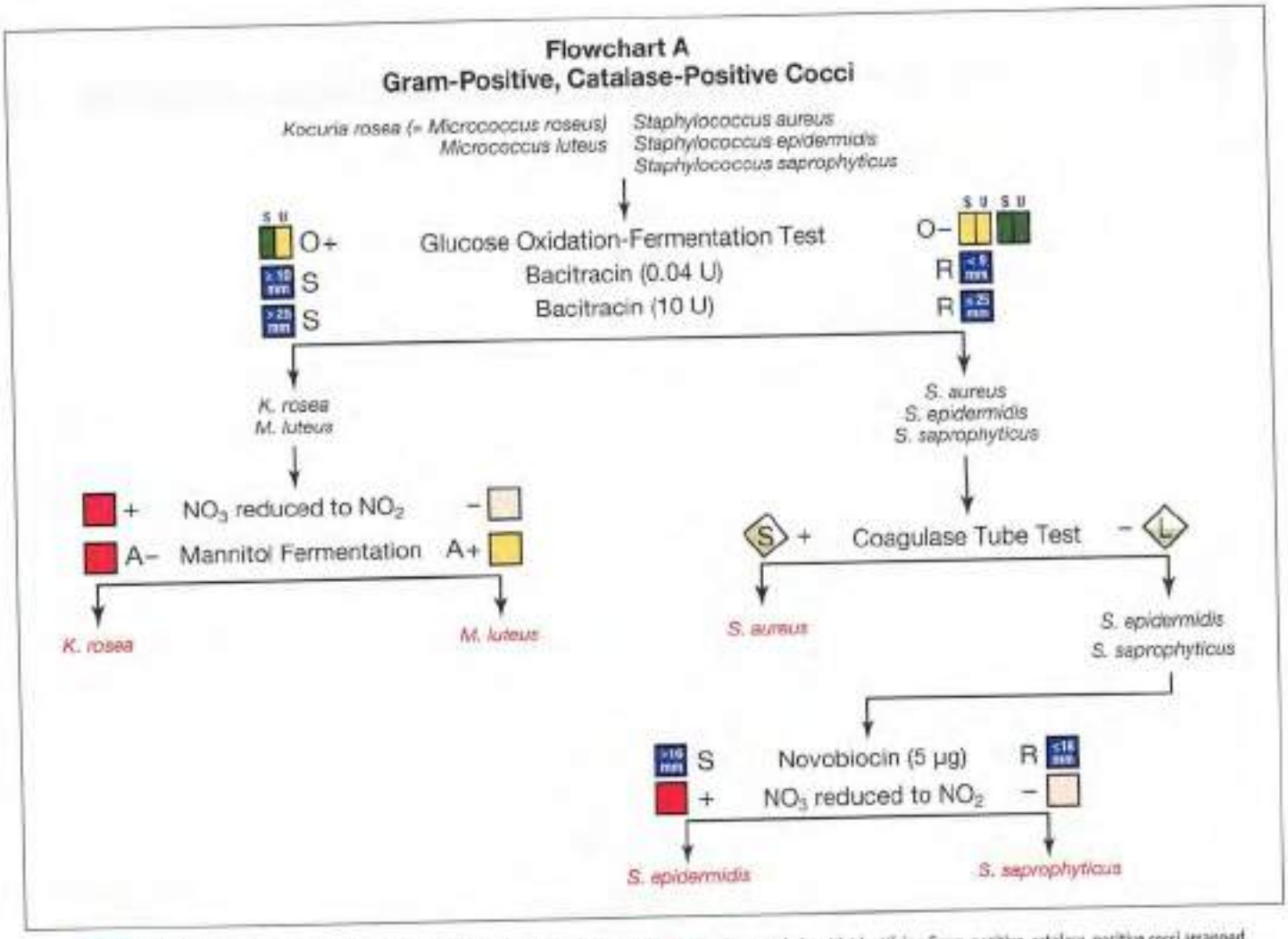


FIGURE **H.2** **Identification Flowchart A (Gram-Positive, Catalase-Positive Cocci)** • This chart can help with identifying Gram-positive, catalase-positive cocci arranged mainly in tetrads or clusters. See the exercise(s) as listed in Figure H.1 for instructions on how to run and interpret each test. In some cases, final identification to species is difficult. Try to get that far, but you may reach a point where your lab strains cannot be differentiated any further.

H

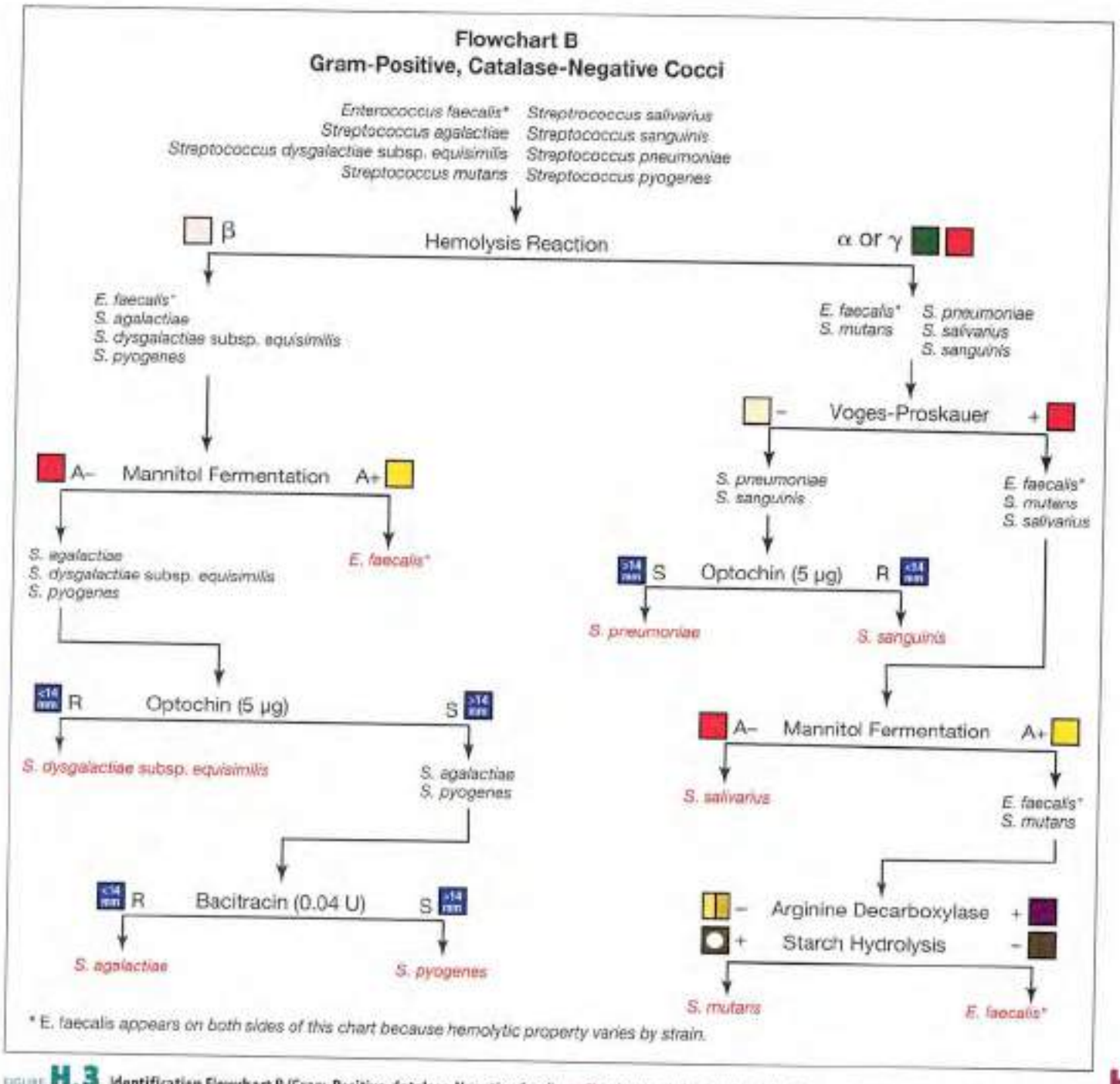


FIGURE **H.3** **Identification Flowchart B (Gram-Positive, Catalase-Negative Cocci)** • This chart can help with identifying Gram-positive, catalase-negative cocci that are mainly arranged as pairs or in chains. Refer to the exercise(s) as listed in Figure H.1 for details on how to perform and interpret each test.

H

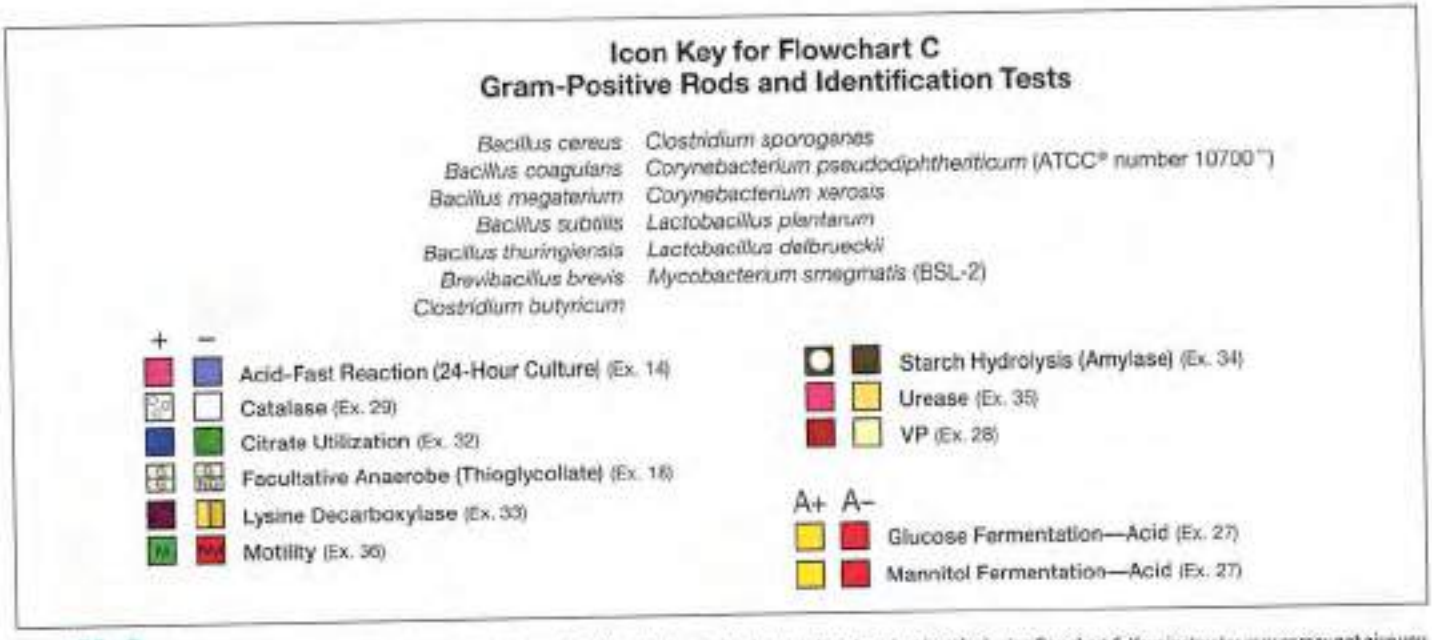


FIGURE **H.4** **Icon Key for Identification Flowchart C for Gram-Positive Rods** • The bacteria listed can be identified using flowchart C. Your instructor may or may not give you an unknown bacterium from this list. If you do have a bacterium from this list, the tests noted may be useful for identifying your unknown. Be aware, the list is not exhaustive, but rather is to provide you with some direction as you try to identify your unknown bacterium. Icon colors are to remind you what the results look like. The icons should not be used to compare with your results. ***Note:*** For the fermentation tests, "A+" means acid is produced; "A−" means acid is not made.

H

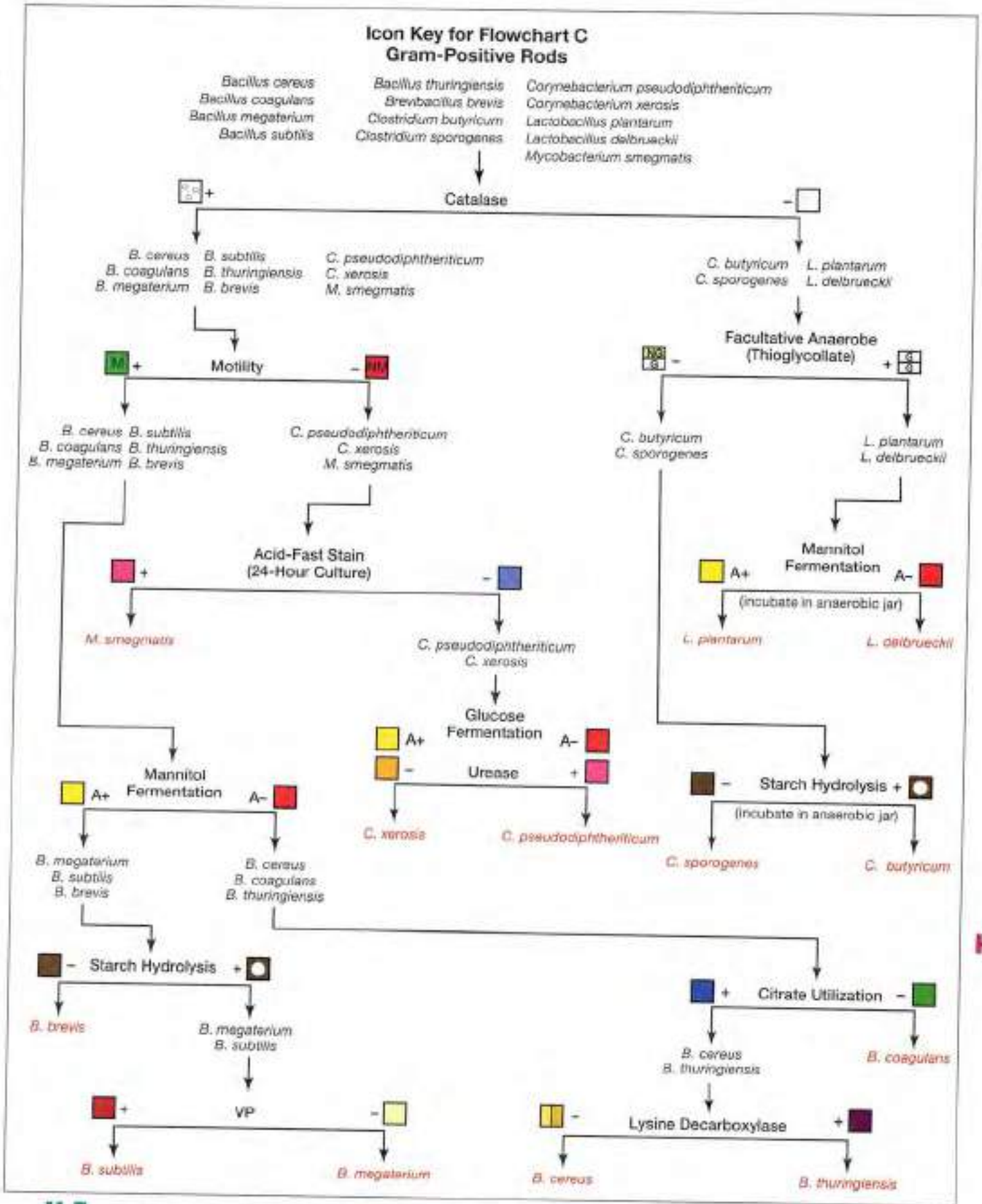


FIGURE **H.5** **Identification Flowchart C (Gram-Positive Rods)** • This flowchart can help with identifying Gram-positive rods. See the exercise(s) as listed in Figure H.4 for instructions on how to run and interpret each test.

— Lab Notes —

APPENDIX I

Identification Flowcharts for *Enterobacteriaceae*

These sample icon keys and flowcharts may be helpful as you conduct the bacterial unknown lab in Exercise 48.

Key for Selecting *Enterobacteriaceae* Identification Flowchart Based on IMViC Results

*Citrobacter freundii**
Citrobacter koseri
Enterobacter aerogenes
Enterobacter cloacae
Escherichia coli
*Hafnia alvei**
*Klebsiella pneumoniae*** (BSL-2)
Morganella morganii (BSL-2)
*Proteus mirabilis** (BSL-2)
Proteus vulgaris (BSL-2)
Providencia alcalifaciens
Providencia stuartii
*Salmonella enterica**** (BSL-2)
Serratia liquefaciens
*Serratia marcescens**
*Shigella flexneri (Group B)** (BSL-2)
Shigella sonnei (Group D) (BSL-2)

*Organism shows up in more than one figure due to variable results of one or more IMViC tests

†*K. pneumoniae* is *K. pneumoniae* subsp. *K. pneumoniae*

††*Salmonella enterica* is *Salmonella enterica* subsp. *enterica* and includes *S. enteritidis* and *S. typhimurium*

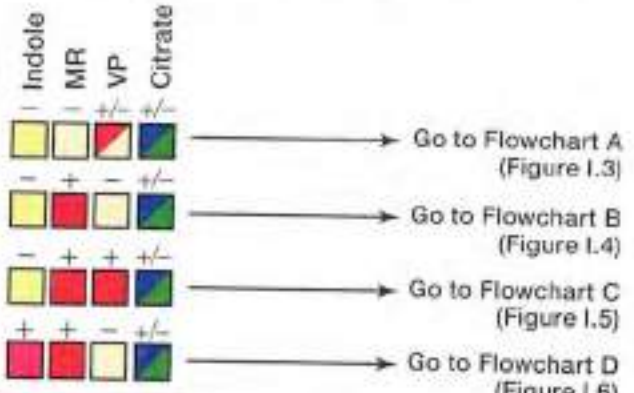


FIGURE I.1 Key for Selecting *Enterobacteriaceae* Identification Flowchart Based on IMViC Results • The bacteria listed can be identified using the noted flowcharts. Your instructor may or may not give you an unknown bacterium from this list. To decide which flowchart would be the most useful, match the IMViC results of your isolate with one of the four shown, and then proceed with the flowchart in the figure indicated. Icon colors are to remind you what the results look like. The icons should not be used to compare with your results. Some organisms are listed in more than one flowchart because of variable results for a particular test. A box divided diagonally with the symbol "+/−" means either result for that test is a match.

FIGURE I.2 Key to Tests and Result Icons Used in Flowcharts A–D to Identify *Enterobacteriaceae* • Icon colors are to remind you what the results look like. The icons should not be used to compare with your results. **Note:** For the fermentation tests, "A+" means acid is produced; "A−" means acid is not made.

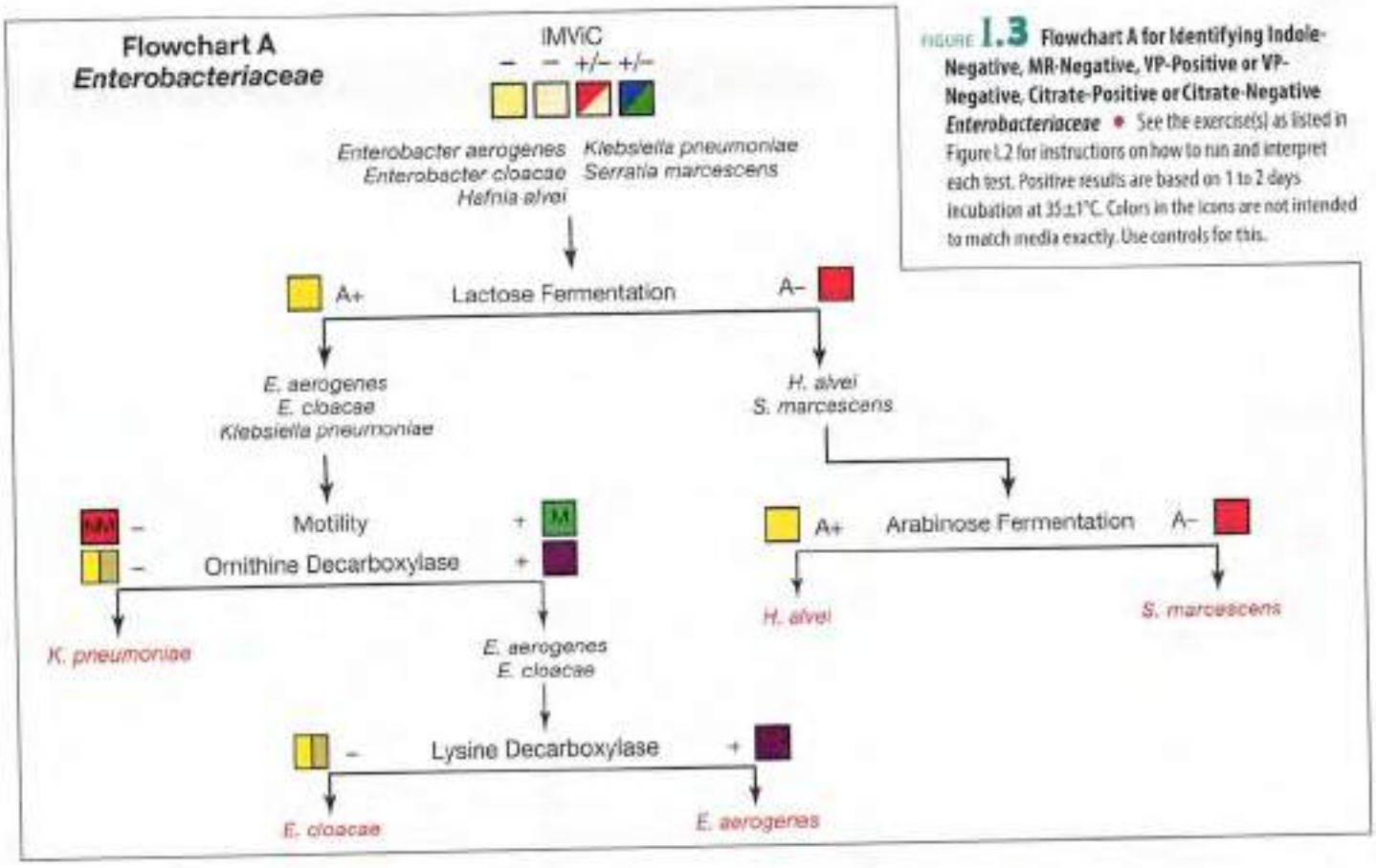


FIGURE **1.3** **Flowchart A for Identifying Indole-Negative, MR-Negative, VP-Positive or VP-Negative, Citrate-Positive or Citrate-Negative *Enterobacteriaceae*** • See the exercise(s) as listed in Figure I.2 for instructions on how to run and interpret each test. Positive results are based on 1 to 2 days incubation at 35±1°C. Colors in the icons are not intended to match media exactly. Use controls for this.

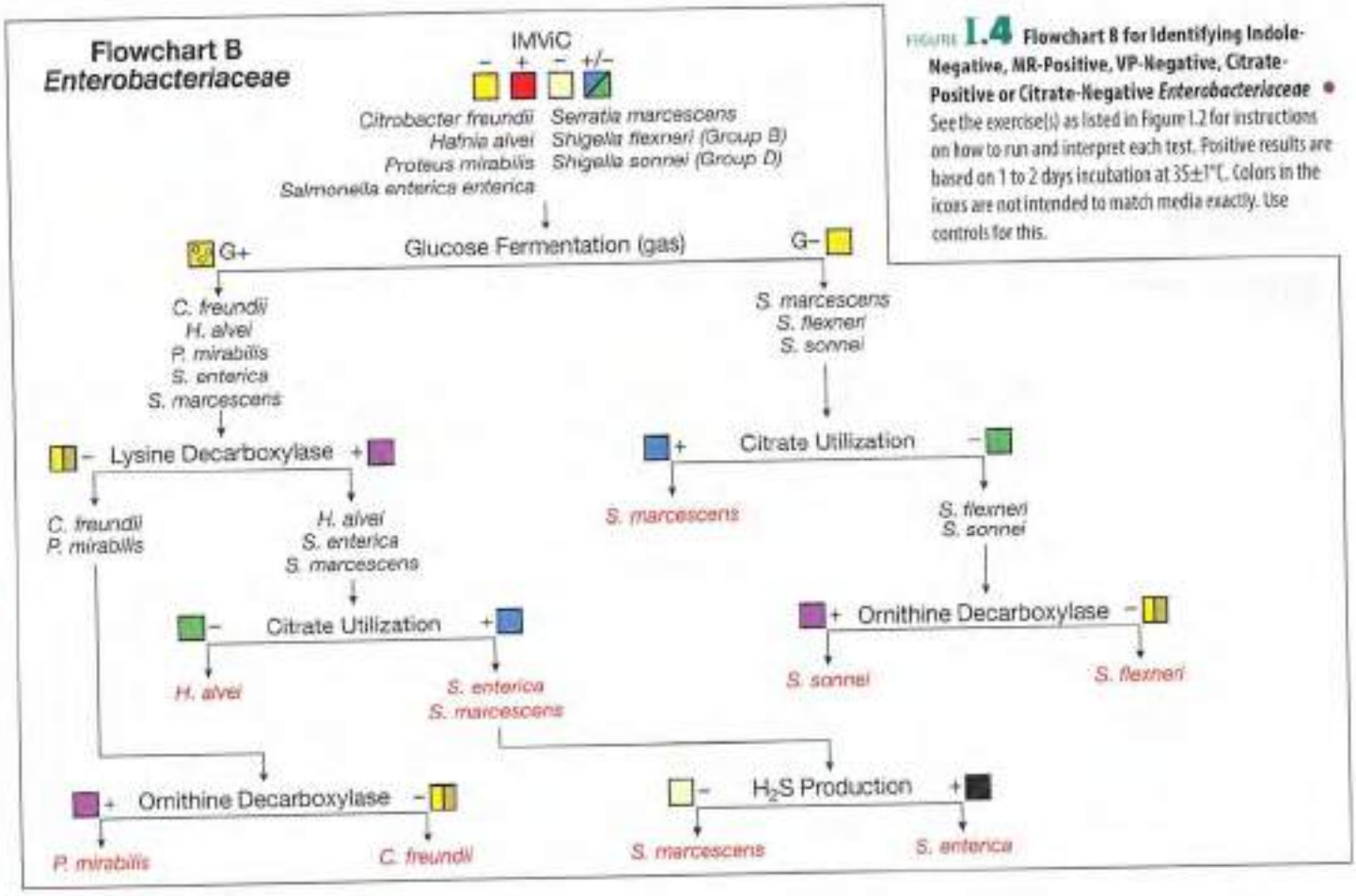


FIGURE **1.4** **Flowchart B for Identifying Indole-Negative, MR-Positive, VP-Negative, Citrate-Positive or Citrate-Negative *Enterobacteriaceae*** • See the exercise(s) as listed in Figure I.2 for instructions on how to run and interpret each test. Positive results are based on 1 to 2 days incubation at 35±1°C. Colors in the icons are not intended to match media exactly. Use controls for this.

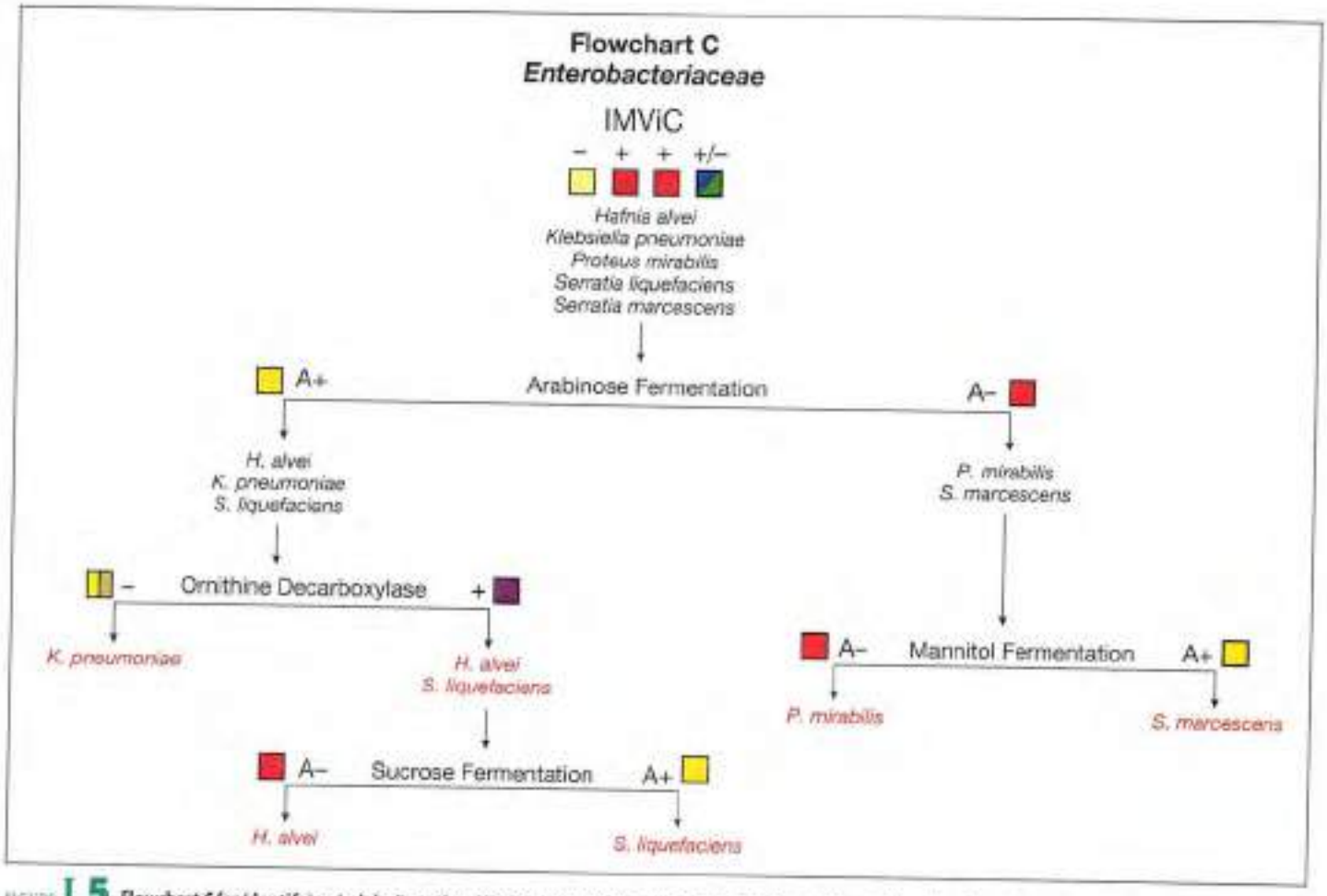


FIGURE **I.5** **Flowchart C for Identifying Indole-Negative, MR-Positive, VP-Positive, Citrate-Positive or Citrate-Negative *Enterobacteriaceae*** • See the exercise(s) as listed in Figure 1.2 for instructions on how to run and interpret each test. Positive results are based on 1 to 2 days incubation at 35±1°C. Colors in the icons are not intended to match media exactly. Use controls for this.

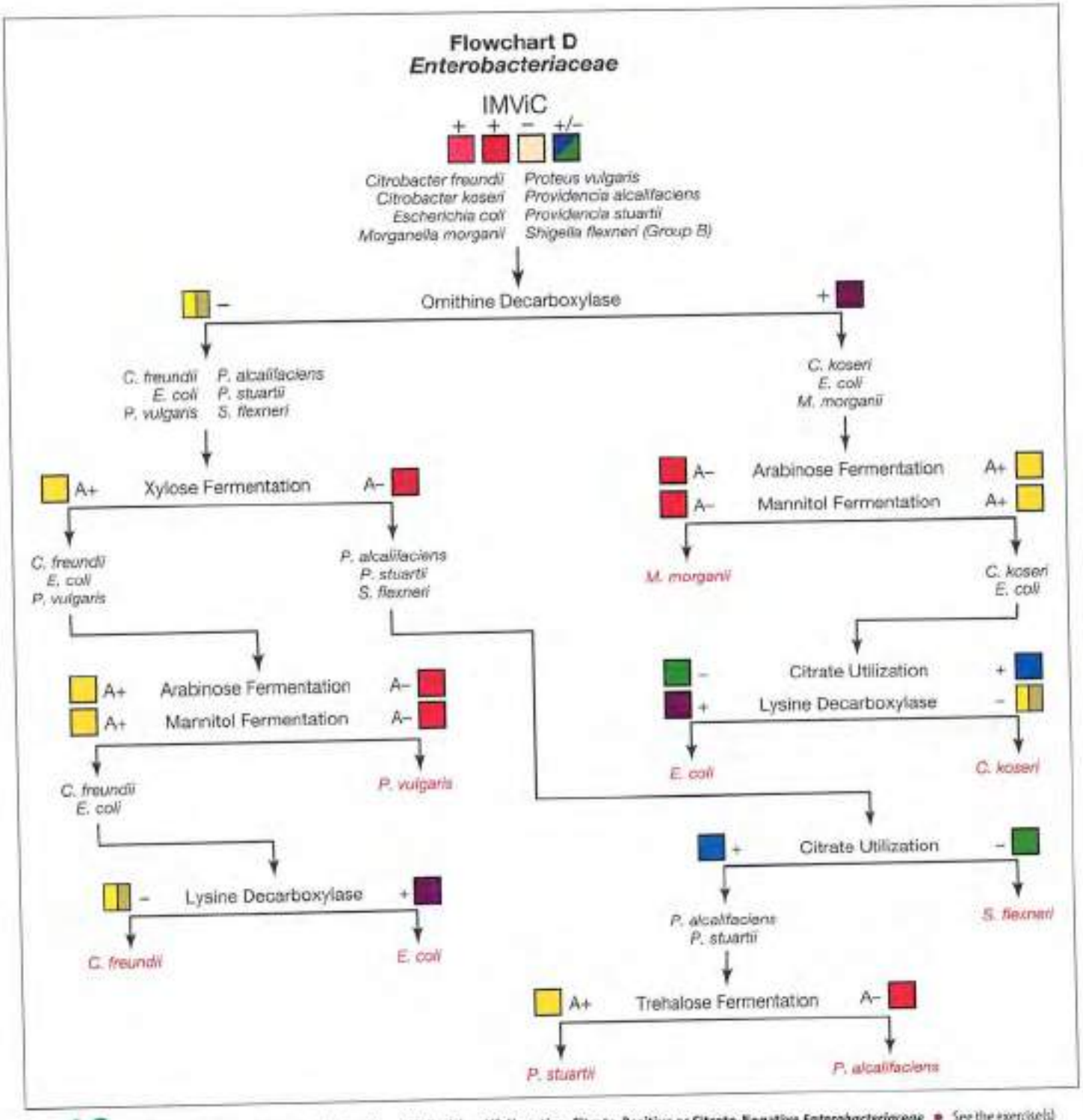


FIGURE **1.6** **Flowchart D for Identifying Indole-Positive, MR-Positive, VP-Negative, Citrate-Positive or Citrate-Negative *Enterobacteriaceae*** • See the exercise(s) as listed in Figure 1.2 for instructions on how to run and interpret each test. Positive results are based on 1 to 2 days incubation at 35±1°C. Colors in the icons are not intended to match media exactly. Use controls for this.

GLOSSARY

A

absorbance A measurement, using a spectrophotometer, of how much light entering a substance is not transmitted.

acetoin A four-carbon intermediate in the conversion of pyruvic acid to 2,3-Butanediol; the compound detected in the Voges-Proskauer test.

acetyl-CoA Compound that enters the Krebs cycle by combining with oxaloacetate to form citrate; may be produced from pyruvate in carbohydrate metabolism or from oxidation of fatty acids.

acidic stain Staining solution with a negatively charged chromophore.

acidophile Microorganism adapted to a habitat below pH 5.5.

adenosine triphosphate (ATP) In hydrolysis of ATP to adenosine diphosphate (ADP), supplies the energy necessary to perform most work in the cell.

aerobe Microorganism that requires oxygen for growth.

aerobic respiration A type of energy releasing metabolism in which oxygen is the final electron acceptor in the pathway. It also has the highest potential ATP yield of all catabolic and exergonic reactions.

aerosol Droplet nuclei that remain suspended in the air for a long time.

aerotolerance Designation of an organism based on its ability to grow in the presence of oxygen.

aerotolerant anaerobe Anaerobe that grows in the presence of oxygen but does not use it metabolically.

alkaliphile Microorganism adapted to a habitat above pH 8.5.

alpha (α) hemolysis The greening around a colony on blood agar as a result of partial destruction of red blood cells; typical of certain members of *Streptococcus*.

amphitrichous Describes a flagellar arrangement with flagella at both ends of an elongated cell.

amylase Family of enzymes that hydrolyze starch.

anaerobe Microorganism that cannot tolerate oxygen.

anaerobic respiration A type of energy releasing metabolism in which an inorganic compound other than oxygen is the final electron acceptor. These include NO_3, NO_2, SO_4, and CO_2. The ATP yield is higher than fermentation, but not as high as aerobic respiration.

antibiotic Refers to an antimicrobial substance produced by a microorganism such as a bacterium or fungus.

antimicrobial/antimicrobic Describes any substance that kills microorganisms, natural or synthetic.

aseptic Describes the condition of being without contamination.

autotroph An organism that is able to make all of its organic molecules using CO_2 as the source of carbon. *See also* heterotroph.

B

bacillus Rod-shaped cell.

basic stain Staining solution with a positively charged chromophore.

β-hemolysis The clearing around a colony on blood agar resulting from complete destruction of red blood cells; typical of certain members of *Streptococcus*.

β-lactamase Enzyme found in some bacteria that breaks a bond in the β-lactam ring of β-lactam antibiotics, rendering the antibiotic ineffective; the source of resistance against these antibiotics.

biofilm A layer of bacterial cells adhering to and reproducing on a surface.

biosafety level (BSL) One of four sets of minimum standards for laboratory practices, facilities, and equipment to be used when handling organisms at each level; BSL-1 requires the least care, BSL-4 the most.

C

capnophile Microaerophile that requires elevated CO_2 levels.

capsule Insoluble, mucoid, extracellular material surrounding some bacteria.

carbohydrate One of four families of biochemicals; characterized by containing carbon, hydrogen, and oxygen in the ratio 1:2:1.

cardinal temperatures Minimum, optimum, and maximum temperatures for an organism.

casein Milk protein that gives milk its white color.

catalase Enzyme produced by some bacteria that catalyzes the breakdown of metabolic H_2O_2.

chemoheterotroph Any organism that uses chemicals for energy and organic compounds as a carbon source. Usually the same organic compounds serve both purposes.

chemolithotroph Any organism that gets its energy from chemicals and its electrons from an inorganic source. No eukaryotes are chemolithotrophic, only some prokaryotes (e.g., sulfur and ammonia-oxidizing bacteria) are chemolithotrophs.

chromogenic reducing agent A substance that produces color when it gives up electrons (becomes oxidized).

chromophore The charged region of a dye molecule that gives it its color.

coagulase *See* bound coagulase; free coagulase.

coagulase-reacting factor Plasma component that reacts with free coagulase to trigger the clotting mechanism.

coccus Spherical cell.

coenzyme Nonprotein portion of an enzyme that aids the catalytic reaction (usually by accepting or donating electrons).

coliform Member of *Enterobacteriaceae* that ferments lactose with production of gas within 48 hours at 37°C.

colony Visible mass of cells produced on culture media from a single cell or single CFU (cell forming unit); a pure culture.

colony forming unit Term used to define the cell or group of cells (e.g., staphylococci, streptococci) that produces a colony when transferred to plated media.

commensal Describes a synergistic relationship between two organisms in which one benefits from the relationship and the other is affected neither negatively nor positively.

common source epidemic An epidemic in which the disease is transmitted from a single source (such as water supply) and is not transmitted person-to-person.

complex medium A medium in which at least one nutritional ingredient is of unknown composition or amount. Media with any plant or animal extract are always complex.

confirmatory test One last test after identification of an unknown, to further support the identification.

conjugate acid A compound (one of a conjugate pair) that donates a proton in solution.

conjugate base A compound (one of a conjugate pair) that accepts a proton in solution.

conjugate pair A compound that alternates between acid and base forms by losing or gaining one proton. Example: HCl/Cl^-.

counterstain Stain applied after decolorization to provide contrast between cells that were decolorized and those that weren't.

culture A liquid or solid medium with microorganisms growing in or on it.

cyst "Resting stage" in life cycle of certain protozoans. *See also* trophozoite.

cytochrome A class of iron-containing enzymes in electron transport chains; characterized by a porphyrin ring structure.

cytochrome oxidase An electron carrier in the electron transport chain of aerobes, facultative anaerobes, and microaerophiles that makes the final transfer of electrons to oxygen.

cytoplasm The semifluid component of cells inside the cellular membrane in which many chemical reactions take place.

D

deaminase Enzyme that catalyzes the removal of the amine group (NH_2) from an amino acid.

decarboxylase Enzyme that catalyzes the removal of the carboxyl group (COOH) from an amino acid.

defined medium A growth medium in which the exact amount and chemical formula (and thus identity) of each ingredient are known.

deoxyribonuclease A family of hydrolytic enzymes that depolymerize DNA into polynucleotides. The precise location of hydrolysis within the sugar-phosphate backbone depends on which DNase is acting. These are usually secreted enzymes.

differential medium Growth medium that contains an indicator (usually color) to detect the presence or absence of a specific metabolic activity.

differential stain Staining procedure that allows distinction between cell types or parts of cells; often involves more than one stain, but not necessarily.

dilution blank A test tube containing a measured volume of sterile diluent (water, saline, or buffer) used to serially dilute a concentrated solution or broth.

dilution factor Proportion of original sample present in a new mixture after it has been diluted; calculated by dividing the volume of the original sample by the total volume of the new mixture. (Subsequent dilutions in a serial dilution must be multiplied by the dilution factor of the previous dilution.)

diploid Defines a cell that has two complete sets of genetic information; one stage in the life cycle of all eukaryotic microorganisms (which also have a haploid [one set] stage).

disaccharide A sugar made of two monosaccharide subunits; e.g., the monosaccharides glucose and galactose can combine to make the disaccharide lactose.

disk diffusion test (Kirby-Bauer Test) This is one way to check susceptibility and resistance of a microbe to an antibiotic. A paper disk containing an antibiotic is placed on a plate inoculated to produce confluent growth of a bacterium. Susceptibility is determined by the size of the zone of inhibition of growth around the disk.

Durham tube A small, inverted test tube used in some liquid media to trap gas bubbles and indicate gas production.

E

electromagnetic energy The energy that exists in the form of waves (including x-rays, ultraviolet, visible light, and radio waves).

electron donor A compound that can be oxidized to transfer electrons to another compound, which in turn becomes reduced.

electron transport chain A series of membrane-bound electron carriers that participate in oxidation-reduction reactions whereby electrons are transferred from one carrier to another until given to the final electron acceptor (oxygen in aerobes, other inorganic substances in anaerobes) in respiration.

endergonic Any metabolic reaction in which the products have more potential energy than the reactants. These are usually associated with anabolic (synthesis) reactions.

endospore Dormant, highly resistant form of bacterium; produced only by species of *Bacillus*, *Clostridium*, and a few others.

enteric bacteria Informal name given to bacteria that occupy the intestinal tract.

Enterobacteriaceae A group of Gram-negative rods that also are oxidase-negative, ferment glucose to acid, have polar flagella if motile, and usually are catalase-positive and reduce nitrate to nitrite.

enzyme A protein that catalyzes a metabolic reaction by interacting with the reactant(s) specifically; each metabolic reaction has its own enzyme.

eukaryote Type of cell with membranous organelles, including a nucleus, and having 80S ribosomes and many linear molecules of DNA.

exergonic Any metabolic reaction in which the reactants have more potential energy than the products. These are usually associated with catabolic (degradative) reactions.

exoenzyme Enzyme that operates in the external environment after being secreted from the cells.

extracellular Pertains to the region outside a cell.

extreme thermophile Organism that grows best at temperatures above 80°C.

F

facultative anaerobe Microorganism capable of both fermentation and respiration; grows in the presence or absence of oxygen.

facultative thermophile Microorganism that prefers temperatures above 40°C but will grow in lower temperatures.

false negative Test result that is negative when the sample is actually positive; usually a result of lack of sensitivity in the test system.

false positive Test result that is positive when the sample is actually negative; usually a result of lack of specificity of the test system.

fastidious (microorganism) Describes a microorganism with strict physiological requirements; difficult or impossible to grow unless specific conditions are provided.

fatty acid A long chain, organic molecule with a carboxylic acid at one end with the rest of the carbons being bonded to hydrogens.

fermentation Metabolic process in which an organic molecule acts as an electron donor and one or more of its organic products act as the final electron acceptor, marking the end of the metabolic sequence (differs from respiration in that an inorganic substance is not needed to act as final electron acceptor).

final electron acceptor (FEA) The molecule receiving electrons (it becomes reduced) at the end of a metabolic sequence of oxidation/reduction reactions.

flavin adenine dinucleotide (FAD) Coenzyme used in oxidation/reduction reactions that acts as an electron acceptor or donor, respectively.

flavoprotein A flavin-containing protein in the electron transport chain, capable of receiving and transferring electrons as well as entire hydrogen atoms; sometimes bypasses normal route of transfer and reduces oxygen directly, forming hydrogen peroxide and superoxide radicals.

free coagulase An enzyme produced and secreted by some microorganisms that initiates the clotting mechanism in plasma; seen as a solid mass in the coagulase tube test or clumps in the slide test.

free energy (of formation) The energy released by a molecule in a chemical reaction that is able to do work (e.g., produce ATP). It is equivalent to the energy required to form the molecule from its individual elements.

free living A term used to describe nonparasitic organisms.

G

gametangium A structure that produces gametes; used in describing fungi.

gamete Reproductive cell that must undergo fusion with another gamete to continue the life cycle; usually are haploid. *See also* spore.

germ theory Theory holding that diseases and infections are caused by microorganisms; first hypothesized in the 16th century by Girolamo Fracastoro of Verona.

glycolysis Metabolic process by which a glucose molecule is split into two 3-carbon pyruvic acid molecules, producing two ATP (net) and two $NADH_2$ molecules.

group A streptococci α-hemolytic members of the genus *Streptococcus*, characterized by possessing the Lancefield group A antigen; belong to the species *S. pyogenes*, an important human pathogen.

group B streptococci β-hemolytic members of the genus *Streptococcus*, characterized by possessing the Lancefield group B antigen; belong to the species *S. agalactiae*.

group D streptococci Streptococci possessing the Lancefield group D antigen; include *S. bovis* and species of the genus *Enterococcus*.

H

haploid Describes a cell that has one complete set of genetic information; all eukaryotic microorganisms have a haploid stage and a diploid (two sets) stage in their life cycle; all prokaryotes are haploid.

hemoglobin Iron-containing protein of red blood cells that is responsible for binding oxygen.

hemolysin A class of chemicals produced by some bacteria that break down hemoglobin and produce hemolysis.

heterotroph An organism that requires carbon in the form of organic molecules. *See also* autotroph.

host An organism that serves as a habitat for another organism such as a parasite or a commensal.

hydrolysis The metabolic process of splitting a molecule into two parts, adding a hydrogen ion to one part and a hydroxyl ion to the other. (One water molecule is used in the process.)

hypha (*pl.* hyphae) A filament of fungal cells.

I

IMViC Acronym representing the four tests used in identification of *Enterobacteriaceae*: Indole, Methyl Red, Voges-Proskauer, and Citrate.

incubation The process of growing a culture by supplying it with the necessary environmental conditions.

index case First occurrence of an infection or disease that results in an epidemic.

infectious disease A transmissible illness or infection.

inoculum The organisms used to start a new culture or transferred to a new place.

inorganic molecule A molecule that does *not* contain carbon and hydrogen.

intracellular Within the cell; as an intracellular enzyme catalyzing reactions inside the cell.

iron-sulfur protein Iron-containing electron carrier in electron transport chains. Differentiated from cytochromes by the lack of porphyrin ring structure.

isolate (*v.*) The process of separating individual cell types from a mixed culture; (*n.*) the group of cells resulting from isolation.

K

karyogamy The process of nuclear fusion that occurs after fertilization (plasmogamy) in sexual life cycles.

Krebs cycle A cyclic metabolic pathway found in organisms that respire aerobically or anaerobically.

L

ligate To join together.

limit of resolution The closest two points can be together for the microscope lens to make them appear separate; two points closer than the limit of resolution will blur together.

lipid A fat.

lophotrichous Describes a flagellar arrangement with a group of flagella at one end of an elongated cell.

M

medium A substance used for growing microbes; may be liquid (usually a broth) or solid (usually agar).

meiosis The process in which the nucleus of a diploid eukaryotic cell divides to make four haploid nuclei.

mesophile A microorganism that grows best at temperatures between 15°C and 45°C.

microaerophile A microorganism that requires oxygen, but can't tolerate atmospheric levels of it.

minimum inhibitory concentration (MIC) The lowest concentration of an antimicrobial substance required to inhibit growth of all microbial cells it contacts; on an agar plate, typically the outer edge of the zone of inhibition where the substance has diffused to the degree that it no longer inhibits growth.

mitosis The process in which a nucleus divides to produce two identical nuclei.

mixed acid fermentation Vigorous fermentation producing many acid products including lactic acid, acetic acid, succinic acid, and formic acid, and subsequently lowering the pH of the medium to pH 4.4 or below.

mold Informal grouping of filamentous fungi. *See also* yeast.

monotrichous Describes a flagellar arrangement consisting of a single flagellum.

morbidity Epidemiological measurement of incidence of a disease; typically accompanied by "incidence rate," referring to the incidence of a disease over time.

morphology The shape of an organism.

mortality Epidemiological measurement of death caused by a disease; typically accompanied by "incidence rate," referring to the incidence of death from a disease over time.

mutagen A substance that causes mutation in DNA; most mutagens are carcinogens.

mutation Alteration in a cell's DNA.

mutualistic Describes a synergistic relationship between two organisms in which both benefit from the interaction.

mycelium A mass of fungal filaments (hyphae).

N

neutrophile Microorganism adapted to a habitat between pH 5.5 and 8.5.

nicotinamide adenine dinucleotide (NAD) A coenzyme used in oxidation/reduction reactions that acts as an electron acceptor or donor, respectively.

nitrate A highly oxidized form of nitrogen; NO_3.

nitrate reductase An enzyme produced by all members of *Enterobacteriaceae* (and others) that catalyzes the reduction of nitrate (NO_3) to nitrite (NO_2).

nitrification The chemical process in which nitrogen compounds, such as ammonia and nitrite are oxidized to form nitrate. Typically, different bacteria perform these two steps in which ammonia is first oxidized to nitrite, which is subsequently oxidized to nitrate.

nitrite NO_2, an oxidized form of nitrogen.

noninfectious diseases Conditions not caused by microorganisms; examples are stroke, heart disease, and emphysema.

O

objective lens The microscope lens that first produces magnification of the specimen in a compound microscope.

obligate (strict) aerobe Microorganism that requires oxygen to survive and grow.

obligate (strict) anaerobe Microorganism for which oxygen is lethal; requires the complete absence of oxygen.

obligate thermophile Microorganism that grows only at temperatures above 40°C.

ocular lens The lens the microscopist looks through; produces the virtual image by magnifying the real image.

ocular micrometer A uniformly graduated linear scale placed in the microscope ocular used for measuring microscopic specimens.

opportunistic pathogen A microorganism not ordinarily thought of as pathogenic (i.e., most enterics) that will cause infection when out of its normal habitat.

organic molecule A molecule made of reduced carbon—that is, containing at least carbon and hydrogen.

osmosis Diffusion of water across a semipermeable membrane.

oxidation/reduction Chemical reaction in which electrons are transferred. (The molecule losing the electrons becomes oxidized; the molecule gaining the electrons becomes reduced.)

oxidative deamination The metabolic process in which an amino acid has its amine removed, producing ammonia and an organic acid.

oxidative phosphorylation The process by which an electron transport chain is used to add a phosphate to ADP to make ATP.

oxidizing agent A substance that removes electrons from (oxidizes) another. *See also* reducing agent.

P

parasite An organism that lives symbiotically with another, but to the detriment of the other organism (called a host).

peptidoglycan The insoluble, porous, cross-linked polymer comprising bacterial cell walls; generally thick in Gram-positive organisms and thin in Gram-negative organisms.

peptone A digest of protein used in formulating some bacteriological media.

peritrichous Describes a flagellar arrangement in which flagella arise from the entire surface of the cell.

pH The measure of a solution's alkalinity or acidity; the negative logarithm of the hydrogen ion concentration.

plasma The noncellular (fluid) portion of blood; consists of serum (including serum proteins) and clotting proteins.

plasmid Small, circular, extrachromosomal piece of DNA found in prokaryotic cells; often carries genes for antibiotic resistance.

plasmogamy The cytoplasmic fusion of gametes at the time of fertilization. *See also* karyogamy.

polar flagellum A single flagellum at one end of an elongated cell.

precipitate An insoluble material that comes out of a solution during a precipitation reaction.

presumptive identification Tentative identification of an isolate based on one or more key test results.

primary stain The first stain applied in many differential staining techniques; usually subjected to a decolorization step that forms the basis for the differential stain.

prokaryote Type of cell lacking internal compartmentalization (membranous organelles, including a nucleus) and having 70S ribosomes and a circular molecule of DNA.

propagated transmission Conveying a disease person-to-person.

proteolytic Refers to catabolism of protein; e.g., a proteolytic enzyme.

pseudohypha A chain of fungal cells produced by budding (rather than by cytokinesis that produces two equally sized cells) and which is characterized by constrictions at cell junctions.

psychrophile A microorganism that grows only at temperatures below 20°C, with an optimum around 15°C.

psychrotroph A microorganism that grows optimally at temperatures between 20 and 30°C but will grow at temperatures as low as 0°C and as high as 35°C.

pure culture Microbial culture containing only a single species.

putrefaction The process of digesting dead organic material; decay.

pyruvic acid (pyruvate) A three-carbon compound produced at the end of glycolysis that may enter a respiration or a fermentation pathway; also serves as a starting point for synthesis of certain amino acids and an entry point for their digestion.

Q

quinoidal (compound) A color-producing compound containing quinone as its central structure.

R

real image Magnified image of a specimen produced by the objective lens of a microscope; the real image is magnified again by the ocular lens to produce the virtual image.

reducing agent A substance that donates electrons to (reduces) another. *See also* oxidizing agent.

reductase An enzyme that catalyzes the transfer of electrons from donor molecule to acceptor molecule, thereby reducing the acceptor.

reduction *See* oxidation/reduction.

refraction The bending of light as it passes from a medium with one refractive index into another medium with a different refractive index.

reservoir A nonhuman host or other site in nature serving as a perpetual source of pathogenic organisms.

resolution The clarity of an image produced by a lens; the ability of a lens to distinguish between two points in a specimen; high resolution in a microscope is desirable.

resolving power *See* limit of resolution.

respiration Metabolic process by which an organic molecule acts as an electron donor and an inorganic substance—such as oxygen, sulfur, or nitrate—acts as the final electron acceptor in an electron transport chain, marking the end of the metabolic sequence (differs from fermentation, which uses one of its own organic products as the final electron acceptor).

reversion In carbohydrate fermentation tests, the phenomenon of a microorganism fermentively depleting the carbohydrate and reverting to amino acid metabolism, thereby neutralizing acid products with alkaline products; produces a false negative.

rhizoid A root-like structure used for attachment of some fungi to the substrate.

S

saprophyte A heterotroph that digests dead organic matter; a decomposer.

selective medium Growth medium that favors growth of one group of microorganisms and inhibits or prevents growth of others.

sensitivity This is a measure of a test's ability to detect small amounts of the item being tested for. The better the sensitivity, the fewer the false negatives (due to smaller amounts triggering a positive reaction), and the more useful the test is.

serial dilution Series of dilutions used to reduce the concentration of a culture and thereby produce between 30 and 300 colonies when plated, providing a means of calculating the original concentration.

serum Fluid portion of blood minus the clotting factors.

solute The dissolved substance in a solution.

solution The mixture of dissolved substance (solute) and solvent (liquid).

solvent The liquid portion of a solution in which solute is dissolved.

specificity This is a measure of a test's ability to produce a positive response only when reacting with the particular item being tested for. The better the test's specificity, the fewer the false positives (due to better discrimination), and the more useful it is.

spirillum Spiral-shaped cell.

sporangium Structure that produces spores.

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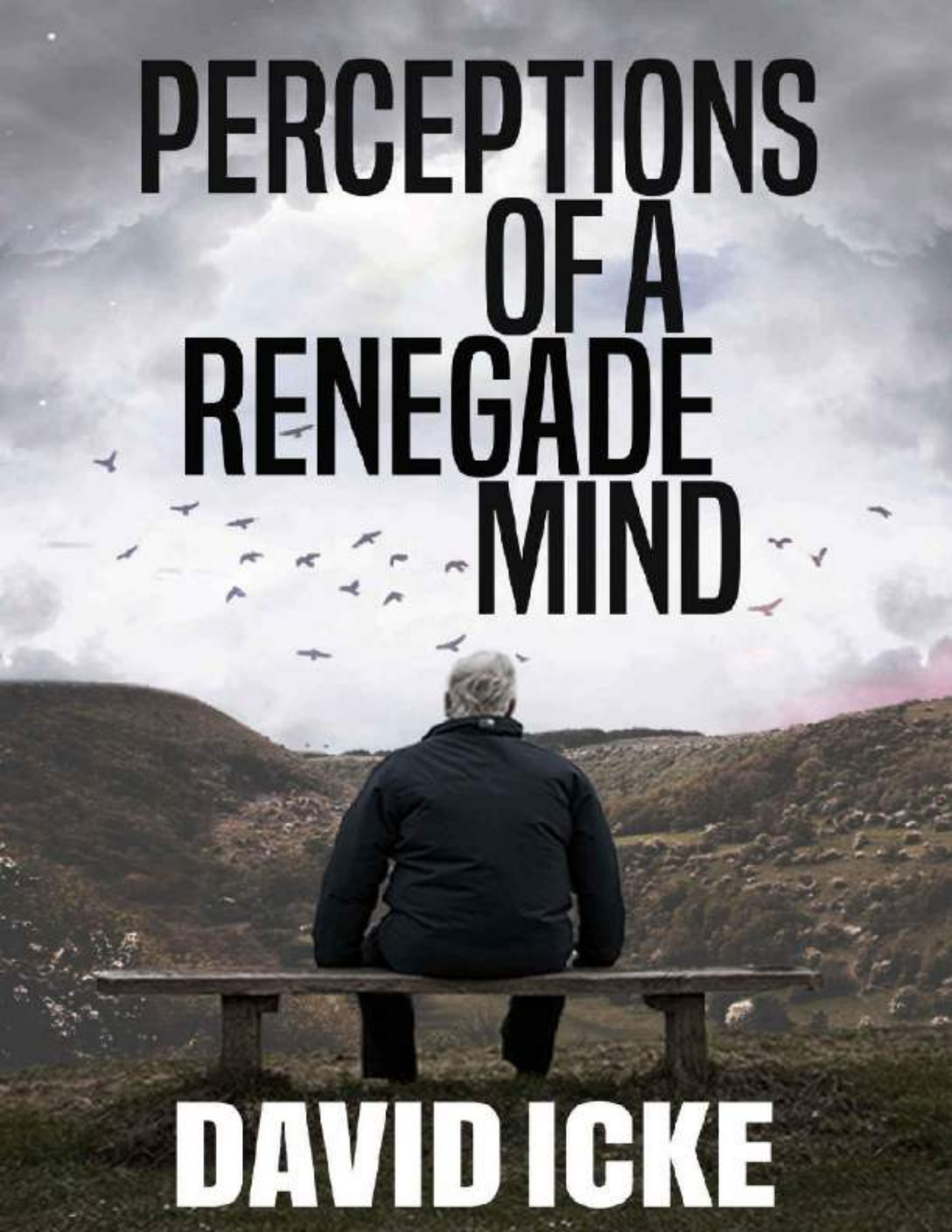

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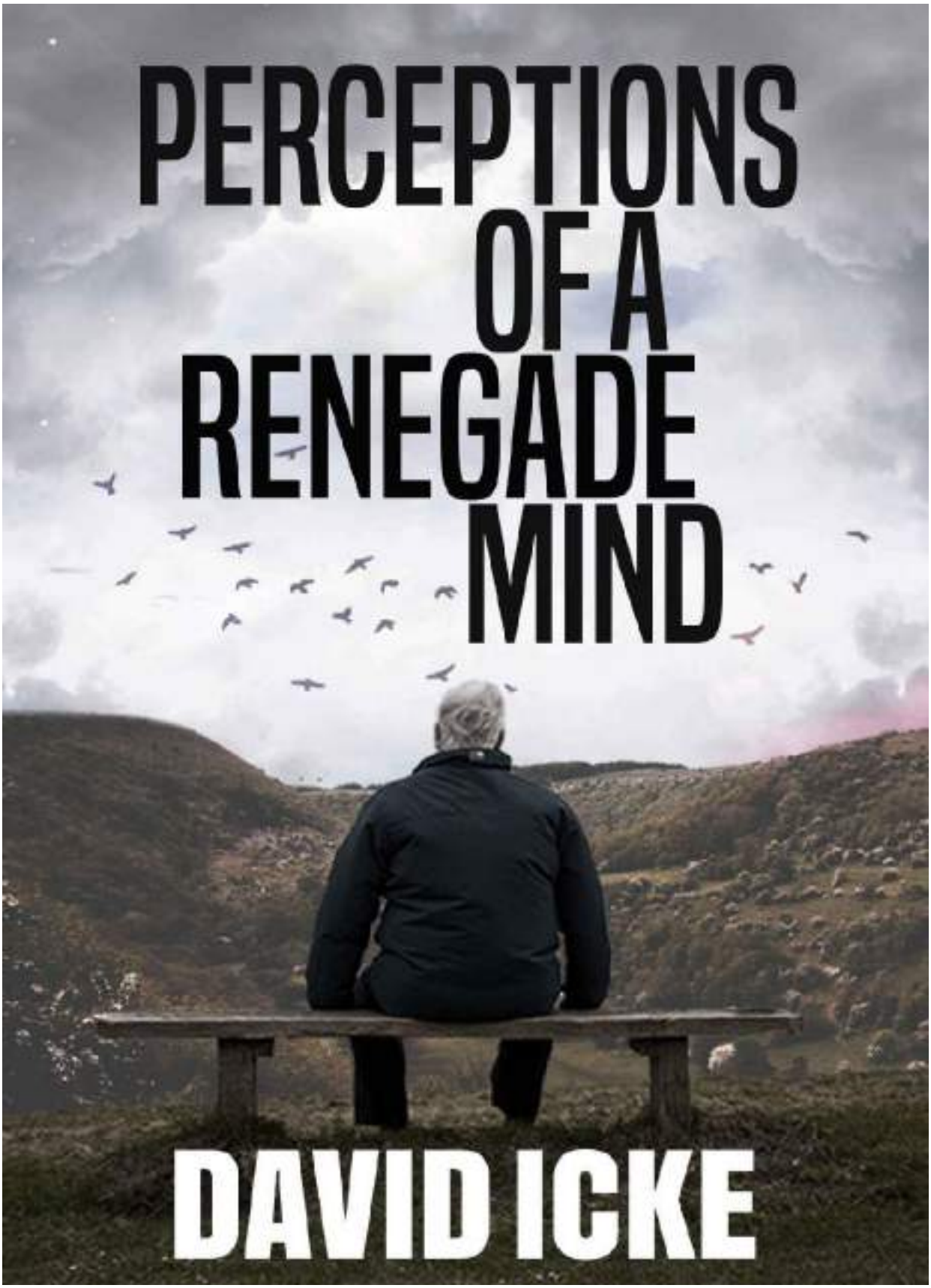
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Renegade:
Adjective
‘Having rejected tradition: Unconventional.’
Merriam-Webster Dictionary

Acquiescence to tyranny is the death of the spirit

You may be 38 years old, as I happen to be. And one day, some great opportunity stands before you and calls you to stand up for some great principle, some great issue, some great cause. And you refuse to do it because you are afraid … You refuse to do it because you want to live longer … You're afraid that you will lose your job, or you are afraid that you will be criticised or that you will lose your popularity, or you're afraid that somebody will stab you, or shoot at you or bomb your house; so you refuse to take the stand.

Well, you may go on and live until you are 90, but you're just as dead at 38 as you would be at 90. And the cessation of breathing in your life is but the belated announcement of an earlier death of the spirit.

Martin Luther King

How the few control the many and always have – the many do whatever they're told

'Forward, the Light Brigade!'
Was there a man dismayed?
Not though the soldier knew
Someone had blundered.
Theirs not to make reply,
Theirs not to reason why,
Theirs but to do and die.
Into the valley of Death
Rode the six hundred.

Cannon to right of them,
Cannon to left of them,
Cannon in front of them
Volleyed and thundered;
Stormed at with shot and shell,
Boldly they rode and well,
Into the jaws of Death,
Into the mouth of hell
Rode the six hundred

Alfred Lord Tennyson (1809-1892)

The mist is lifting slowly
I can see the way ahead
And I've left behind the empty streets
That once inspired my life
And the strength of the emotion
Is like thunder in the air
'Cos the promise that we made each other
Haunts me to the end

The secret of your beauty
And the mystery of your soul
I've been searching for in everyone I meet
And the times I've been mistaken
It's impossible to say
And the grass is growing
Underneath our feet

The words that I remember
From my childhood still are true
That there's none so blind
As those who will not see
And to those who lack the courage
And say it's dangerous to try
Well they just don't know
That love eternal will not be denied

I know you're out there somewhere
Somewhere, somewhere
I know you're out there somewhere

Somewhere you can hear my voice
I know I'll find you somehow
Somehow, somehow
I know I'll find you somehow
And somehow I'll return again to you

The Moody Blues

Are you a gutless wonder - or a Renegade Mind?

Monuments put from pen to paper,
Turns me into a gutless wonder,
And if you tolerate this,
Then your children will be next.
Gravity keeps my head down,
Or is it maybe shame ...

Manic Street Preachers

Rise like lions after slumber
In unvanquishable number.
Shake your chains to earth like dew
Which in sleep have fallen on you.
Ye are many – they are few.

Percy Shelley

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CHAPTER ONE

I'm thinking' – Oh, but *are* you?

Think for yourself and let others enjoy the privilege of doing so too
Voltaire

French-born philosopher, mathematician and scientist René Descartes became famous for his statement in Latin in the 17th century which translates into English as: 'I think, therefore I am.'

On the face of it that is true. Thought reflects perception and perception leads to both behaviour and self-identity. In that sense 'we' are what we think. But who or what is doing the thinking and is thinking the only route to perception? Clearly, as we shall see, 'we' are not always the source of 'our' perception, indeed with regard to humanity as a whole this is rarely the case; and thinking is far from the only means of perception. Thought is the village idiot compared with other expressions of consciousness that we all have the potential to access and tap into. This has to be true when we *are* those other expressions of consciousness which are infinite in nature. We have forgotten this, or, more to the point, been manipulated to forget.

These are not just the esoteric musings of the navel. The whole foundation of human control and oppression is control of perception. Once perception is hijacked then so is behaviour which is dictated by perception. Collective perception becomes collective behaviour and collective behaviour is what we call human society. Perception is all and those behind human control know that which is

why perception is the target 24/7 of the psychopathic manipulators that I call the Global Cult. They know that if they dictate perception they will dictate behaviour and collectively dictate the nature of human society. They are further aware that perception is formed from information received and if they control the circulation of information they will to a vast extent direct human behaviour. Censorship of information and opinion has become globally Nazi-like in recent years and never more blatantly than since the illusory 'virus pandemic' was triggered out of China in 2019 and across the world in 2020. Why have billions submitted to house arrest and accepted fascistic societies in a way they would have never believed possible? Those controlling the information spewing from government, mainstream media and Silicon Valley (all controlled by the same Global Cult networks) told them they were in danger from a 'deadly virus' and only by submitting to house arrest and conceding their most basic of freedoms could they and their families be protected. This monumental and provable lie became the *perception* of the billions and therefore the *behaviour* of the billions. In those few words you have the whole structure and modus operandi of human control. Fear is a perception – **F**alse **E**motion **A**ppearing **R**eal – and fear is the currency of control. In short … get them by the balls (or give them the impression that you have) and their hearts and minds will follow. Nothing grips the dangly bits and freezes the rear-end more comprehensively than fear.

World number 1

There are two 'worlds' in what appears to be one 'world' and the prime difference between them is knowledge. First we have the mass of human society in which the population is maintained in coldly-calculated ignorance through control of information and the 'education' (indoctrination) system. That's all you really need to control to enslave billions in a perceptual delusion in which what are perceived to be *their* thoughts and opinions are ever-repeated mantras that the system has been downloading all their lives through 'education', media, science, medicine, politics and academia

in which the personnel and advocates are themselves overwhelmingly the perceptual products of the same repetition. Teachers and academics in general are processed by the same programming machine as everyone else, but unlike the great majority they never leave the 'education' program. It gripped them as students and continues to grip them as programmers of subsequent generations of students. The programmed become the programmers – the programmed programmers. The same can largely be said for scientists, doctors and politicians and not least because as the American writer Upton Sinclair said: 'It is difficult to get a man to understand something when his salary depends upon his not understanding it.' If your career and income depend on thinking the way the system demands then you will – bar a few free-minded exceptions – concede your mind to the Perceptual Mainframe that I call the Postage Stamp Consensus. This is a tiny band of perceived knowledge and possibility 'taught' (downloaded) in the schools and universities, pounded out by the mainstream media and on which all government policy is founded. Try thinking, and especially speaking and acting, outside of the 'box' of consensus and see what that does for your career in the Mainstream Everything which bullies, harasses, intimidates and ridicules the population into compliance. Here we have the simple structure which enslaves most of humanity in a perceptual prison cell for an entire lifetime and I'll go deeper into this process shortly. Most of what humanity is taught as fact is nothing more than programmed belief. American science fiction author Frank Herbert was right when he said: 'Belief can be manipulated. Only knowledge is dangerous.' In the 'Covid' age belief is promoted and knowledge is censored. It was always so, but never to the extreme of today.

World number 2

A 'number 2' is slang for 'doing a poo' and how appropriate that is when this other 'world' is doing just that on humanity every minute of every day. World number 2 is a global network of secret societies and semi-secret groups dictating the direction of society via

governments, corporations and authorities of every kind. I have spent more than 30 years uncovering and exposing this network that I call the Global Cult and knowing its agenda is what has made my books so accurate in predicting current and past events. Secret societies are secret for a reason. They want to keep their hoarded knowledge to themselves and their chosen initiates and to hide it from the population which they seek through ignorance to control and subdue. The whole foundation of the division between World 1 and World 2 is *knowledge*. What number 1 knows number 2 must not. Knowledge they have worked so hard to keep secret includes (a) the agenda to enslave humanity in a centrally-controlled global dictatorship, and (b) the nature of reality and life itself. The latter (b) must be suppressed to allow the former (a) to prevail as I shall be explaining. The way the Cult manipulates and interacts with the population can be likened to a spider's web. The 'spider' sits at the centre in the shadows and imposes its will through the web with each strand represented in World number 2 by a secret society, satanic or semi-secret group, and in World number 1 – the world of the seen – by governments, agencies of government, law enforcement, corporations, the banking system, media conglomerates and Silicon Valley (Fig 1 overleaf). The spider and the web connect and coordinate all these organisations to pursue the same global outcome while the population sees them as individual entities working randomly and independently. At the level of the web governments *are* the banking system *are* the corporations *are* the media *are* Silicon Valley *are* the World Health Organization working from their inner cores as one unit. Apparently unconnected countries, corporations, institutions, organisations and people are on the *same team* pursuing the same global outcome. Strands in the web immediately around the spider are the most secretive and exclusive secret societies and their membership is emphatically restricted to the Cult inner-circle emerging through the generations from particular bloodlines for reasons I will come to. At the core of the core you would get them in a single room. That's how many people are dictating the direction of human society and its transformation

through the ‘Covid’ hoax and other means. As the web expands out from the spider we meet the secret societies that many people will be aware of – the Freemasons, Knights Templar, Knights of Malta, Opus Dei, the inner sanctum of the Jesuit Order, and such like. Note how many are connected to the Church of Rome and there is a reason for that. The Roman Church was established as a revamp, a rebranding, of the relocated ‘Church’ of Babylon and the Cult imposing global tyranny today can be tracked back to Babylon and Sumer in what is now Iraq.

Figure 1: The global web through which the few control the many. (Image Neil Hague.)

Inner levels of the web operate in the unseen away from the public eye and then we have what I call the cusp organisations located at the point where the hidden meets the seen. They include a series of satellite organisations answering to a secret society founded in London in the late 19th century called the Round Table and among them are the Royal Institute of International Affairs (UK, founded in 1920); Council on Foreign Relations (US, 1921); Bilderberg Group (worldwide, 1954); Trilateral Commission (US/worldwide, 1972); and the Club of Rome (worldwide, 1968) which was created to exploit environmental concerns to justify the centralisation of global power to ‘save the planet’. The Club of Rome instigated with others the human-caused climate change hoax which has led to all the ‘green

new deals' demanding that very centralisation of control. Cusp organisations, which include endless 'think tanks' all over the world, are designed to coordinate a single global policy between political and business leaders, intelligence personnel, media organisations and anyone who can influence the direction of policy in their own sphere of operation. Major players and regular attenders will know what is happening – or some of it – while others come and go and are kept overwhelmingly in the dark about the big picture. I refer to these cusp groupings as semi-secret in that they can be publicly identified, but what goes on at the inner-core is kept very much 'in house' even from most of their members and participants through a fiercely-imposed system of compartmentalisation. Only let them know what they need to know to serve your interests and no more. The structure of secret societies serves as a perfect example of this principle. Most Freemasons never get higher than the bottom three levels of 'degree' (degree of knowledge) when there are 33 official degrees of the Scottish Rite. Initiates only qualify for the next higher 'compartment' or degree if those at that level choose to allow them. Knowledge can be carefully assigned only to those considered 'safe'. I went to my local Freemason's lodge a few years ago when they were having an 'open day' to show how cuddly they were and when I chatted to some of them I was astonished at how little the rank and file knew even about the most ubiquitous symbols they use. The mushroom technique – keep them in the dark and feed them bullshit – applies to most people in the web as well as the population as a whole. Sub-divisions of the web mirror in theme and structure transnational corporations which have a headquarters somewhere in the world dictating to all their subsidiaries in different countries. Subsidiaries operate in their methodology and branding to the same centrally-dictated plan and policy in pursuit of particular ends. The Cult web functions in the same way. Each country has its own web as a subsidiary of the global one. They consist of networks of secret societies, semi-secret groups and bloodline families and their job is to impose the will of the spider and the global web in their particular country. Subsidiary networks control and manipulate the national political system, finance, corporations, media, medicine, etc. to

ensure that they follow the globally-dictated Cult agenda. These networks were the means through which the 'Covid' hoax could be played out with almost every country responding in the same way.

The 'Yessir' pyramid

Compartmentalisation is the key to understanding how a tiny few can dictate the lives of billions when combined with a top-down sequence of imposition and acquiescence. The inner core of the Cult sits at the peak of the pyramidal hierarchy of human society (Fig 2 overleaf). It imposes its will – its agenda for the world – on the level immediately below which acquiesces to that imposition. This level then imposes the Cult will on the level below them which acquiesces and imposes on the next level. Very quickly we meet levels in the hierarchy that have no idea there even is a Cult, but the sequence of imposition and acquiescence continues down the pyramid in just the same way. 'I don't know why we are doing this but the order came from "on-high" and so we better just do it.' Alfred Lord Tennyson said of the cannon fodder levels in his poem *The Charge of the Light Brigade*: 'Theirs not to reason why; theirs but to do and die.' The next line says that 'into the valley of death rode the six hundred' and they died because they obeyed without question what their perceived 'superiors' told them to do. In the same way the population capitulated to 'Covid'. The whole hierarchical pyramid functions like this to allow the very few to direct the enormous many. Eventually imposition-acquiescence-imposition-acquiescence comes down to the mass of the population at the foot of the pyramid. If they acquiesce to those levels of the hierarchy imposing on them (governments/law enforcement/doctors/media) a circuit is completed between the population and the handful of super-psychopaths in the Cult inner core at the top of the pyramid. Without a circuit-breaking refusal to obey, the sequence of imposition and acquiescence allows a staggeringly few people to impose their will upon the entirety of humankind. We are looking at the very sequence that has subjugated billions since the start of 2020. Our freedom has not been taken from us. Humanity has given it

away. Fascists do not impose fascism because there are not enough of them. Fascism is imposed by the population acquiescing to fascism. Put another way allowing their perceptions to be programmed to the extent that leads to the population giving their freedom away by giving their perceptions – their mind – away. If this circuit is not broken by humanity ceasing to cooperate with their own enslavement then nothing can change. For that to happen people have to critically think and see through the lies and window dressing and then summon the backbone to act upon what they see. The Cult spends its days working to stop either happening and its methodology is systematic and highly detailed, but it can be overcome and that is what this book is all about.

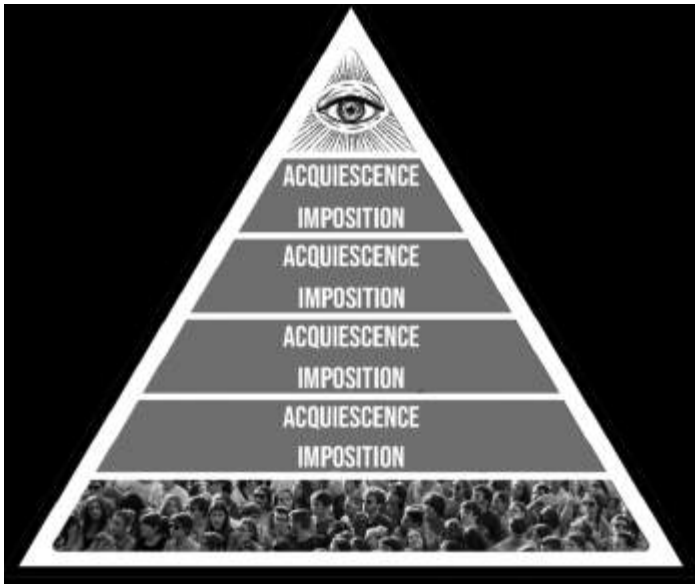

Figure 2: The simple sequence of imposition and compliance that allows a handful of people at the peak of the pyramid to dictate the lives of billions.

The Life Program

Okay, back to world number 1 or the world of the 'masses'. Observe the process of what we call 'life' and it is a perceptual download from cradle to grave. The Cult has created a global structure in which perception can be programmed and the program continually topped-up with what appears to be constant confirmation that the program is indeed true reality. The important word here is 'appears'.

This is the structure, the fly-trap, the Postage Stamp Consensus or Perceptual Mainframe, which represents that incredibly narrow band of perceived possibility delivered by the 'education' system, mainstream media, science and medicine. From the earliest age the download begins with parents who have themselves succumbed to the very programming their children are about to go through. Most parents don't do this out of malevolence and mostly it is quite the opposite. They do what they believe is best for their children and that is what the program has told them is best. Within three or four years comes the major transition from parental programming to full-blown state (Cult) programming in school, college and university where perceptually-programmed teachers and academics pass on their programming to the next generations. Teachers who resist are soon marginalised and their careers ended while children who resist are called a problem child for whom Ritalin may need to be prescribed. A few years after entering the 'world' children are under the control of authority figures representing the state telling them when they have to be there, when they can leave and when they can speak, eat, even go to the toilet. This is calculated preparation for a lifetime of obeying authority in all its forms. Reflex-action fear of authority is instilled by authority from the start. Children soon learn the carrot and stick consequences of obeying or defying authority which is underpinned daily for the rest of their life. Fortunately I daydreamed through this crap and never obeyed authority simply because it told me to. This approach to my alleged 'betters' continues to this day. There can be consequences of pursuing open-minded freedom in a world of closed-minded conformity. I spent a lot of time in school corridors after being ejected from the classroom for not taking some of it seriously and now I spend a lot of time being ejected from Facebook, YouTube and Twitter. But I can tell you that being true to yourself and not compromising your self-respect is far more exhilarating than bowing to authority for authority's sake. You don't have to be a sheep to the shepherd (authority) and the sheep dog (fear of not obeying authority).

The perceptual download continues throughout the formative years in school, college and university while script-reading 'teachers', 'academics' 'scientists', 'doctors' and 'journalists' insist that ongoing generations must be as programmed as they are. Accept the program or you will not pass your 'exams' which confirm your 'degree' of programming. It is tragic to think that many parents pressure their offspring to work hard at school to download the program and qualify for the next stage at college and university. The late, great, American comedian George Carlin said: 'Here's a bumper sticker I'd like to see: We are proud parents of a child who has resisted his teachers' attempts to break his spirit and bend him to the will of his corporate masters.' Well, the best of luck finding many of those, George. Then comes the moment to leave the formal programming years in academia and enter the 'adult' world of work. There you meet others in your chosen or prescribed arena who went through the same Postage Stamp Consensus program before you did. There is therefore overwhelming agreement between almost everyone on the basic foundations of Postage Stamp reality and the rejection, even contempt, of the few who have a mind of their own and are prepared to use it. This has two major effects. Firstly, the consensus confirms to the programmed that their download is really how things are. I mean, everyone knows that, right? Secondly, the arrogance and ignorance of Postage Stamp adherents ensure that anyone questioning the program will have unpleasant consequences for seeking their own truth and not picking their perceptions from the shelf marked: 'Things you must believe without question and if you don't you're a dangerous lunatic conspiracy theorist and a harebrained nutter'.

Every government, agency and corporation is founded on the same Postage Stamp prison cell and you can see why so many people believe the same thing while calling it their own 'opinion'. Fusion of governments and corporations in pursuit of the same agenda was the definition of fascism described by Italian dictator Benito Mussolini. The pressure to conform to perceptual norms downloaded for a lifetime is incessant and infiltrates society right

down to family groups that become censors and condemners of their own 'black sheep' for not, ironically, being sheep. We have seen an explosion of that in the 'Covid' era. Cult-owned global media unleashes its propaganda all day every day in support of the Postage Stamp and targets with abuse and ridicule anyone in the public eye who won't bend their mind to the will of the tyranny. Any response to this is denied (certainly in my case). They don't want to give a platform to expose official lies. Cult-owned-and-created Internet giants like Facebook, Google, YouTube and Twitter delete you for having an unapproved opinion. Facebook boasts that its AI censors delete 97-percent of 'hate speech' before anyone even reports it. Much of that 'hate speech' will simply be an opinion that Facebook and its masters don't want people to see. Such perceptual oppression is widely known as fascism. Even Facebook executive Benny Thomas, a 'CEO Global Planning Lead', said in comments secretly recorded by investigative journalism operation Project Veritas that Facebook is 'too powerful' and should be broken up:

I mean, no king in history has been the ruler of two billion people, but Mark Zuckerberg is … And he's 36. That's too much for a 36-year-old ... You should not have power over two billion people. I just think that's wrong.

Thomas said Facebook-owned platforms like Instagram, Oculus, and WhatsApp needed to be separate companies. 'It's too much power when they're all one together'. That's the way the Cult likes it, however. We have an executive of a Cult organisation in Benny Thomas that doesn't know there is a Cult such is the compartmentalisation. Thomas said that Facebook and Google 'are no longer companies, they're countries'. Actually they are more powerful than countries on the basis that if you control information you control perception and control human society.

I love my oppressor

Another expression of this psychological trickery is for those who realise they are being pressured into compliance to eventually

convince themselves to believe the official narratives to protect their self-respect from accepting the truth that they have succumbed to meek and subservient compliance. Such people become some of the most vehement defenders of the system. You can see them everywhere screaming abuse at those who prefer to think for themselves and by doing so reminding the compliers of their own capitulation to conformity. 'You are talking dangerous nonsense you Covidiot!!' Are you trying to convince me or yourself? It is a potent form of Stockholm syndrome which is defined as: 'A psychological condition that occurs when a victim of abuse identifies and attaches, or bonds, positively with their abuser.' An example is hostages bonding and even 'falling in love' with their kidnappers. The syndrome has been observed in domestic violence, abused children, concentration camp inmates, prisoners of war and many and various Satanic cults. These are some traits of Stockholm syndrome listed at goodtherapy.org:

- Positive regard towards perpetrators of abuse or captor [see 'Covid'].
- Failure to cooperate with police and other government authorities when it comes to holding perpetrators of abuse or kidnapping accountable [or in the case of 'Covid' cooperating with the police to enforce and defend their captors' demands].
- Little or no effort to escape [see 'Covid'].
- Belief in the goodness of the perpetrators or kidnappers [see 'Covid'].
- Appeasement of captors. This is a manipulative strategy for maintaining one's safety. As victims get rewarded – perhaps with less abuse or even with life itself – their appeasing behaviours are reinforced [see 'Covid'].
- Learned helplessness. This can be akin to 'if you can't beat 'em, join 'em'. As the victims fail to escape the abuse or captivity, they may start giving up and soon realize it's just easier for everyone if they acquiesce all their power to their captors [see 'Covid'].

- Feelings of pity toward the abusers, believing they are actually victims themselves. Because of this, victims may go on a crusade or mission to 'save' [protect] their abuser [see the venom unleashed on those challenging the official 'Covid' narrative].
- Unwillingness to learn to detach from their perpetrators and heal. In essence, victims may tend to be less loyal to themselves than to their abuser [*definitely* see 'Covid'].

Ponder on those traits and compare them with the behaviour of great swathes of the global population who have defended governments and authorities which have spent every minute destroying their lives and livelihoods and those of their children and grandchildren since early 2020 with fascistic lockdowns, house arrest and employment deletion to 'protect' them from a 'deadly virus' that their abusers' perceptually created to bring about this very outcome. We are looking at mass Stockholm syndrome. All those that agree to concede their freedom will believe those perceptions are originating in their own independent 'mind' when in fact by conceding their reality to Stockholm syndrome they have by definition conceded any independence of mind. Listen to the 'opinions' of the acquiescing masses in this 'Covid' era and what gushes forth is the repetition of the official version of everything delivered unprocessed, unfiltered and unquestioned. The whole programming dynamic works this way. I must be free because I'm told that I am and so I think that I am.

You can see what I mean with the chapter theme of 'I'm thinking – Oh, but *are* you?' The great majority are not thinking, let alone for themselves. They are repeating what authority has told them to believe which allows them to be controlled. Weaving through this mentality is the fear that the 'conspiracy theorists' are right and this again explains the often hysterical abuse that ensues when you dare to contest the official narrative of anything. Denial is the mechanism of hiding from yourself what you don't want to be true. Telling people what they want to hear is easy, but it's an infinitely greater challenge to tell them what they would rather not be happening.

One is akin to pushing against an open door while the other is met with vehement resistance no matter what the scale of evidence. I don't want it to be true so I'll convince myself that it's not. Examples are everywhere from the denial that a partner is cheating despite all the signs to the reflex-action rejection of any idea that world events in which country after country act in exactly the same way are centrally coordinated. To accept the latter is to accept that a force of unspeakable evil is working to destroy your life and the lives of your children with nothing too horrific to achieve that end. Who the heck wants that to be true? But if we don't face reality the end is duly achieved and the consequences are far worse and ongoing than breaking through the walls of denial today with the courage to make a stand against tyranny.

Connect the dots – but how?

A crucial aspect of perceptual programming is to portray a world in which everything is random and almost nothing is connected to anything else. Randomness cannot be coordinated by its very nature and once you perceive events as random the idea they could be connected is waved away as the rantings of the tinfoil-hat brigade. You can't plan and coordinate random you idiot! No, you can't, but you can hide the coldly-calculated and long-planned behind the *illusion* of randomness. A foundation manifestation of the Renegade Mind is to scan reality for patterns that connect the apparently random and turn pixels and dots into pictures. This is the way I work and have done so for more than 30 years. You look for similarities in people, modus operandi and desired outcomes and slowly, then ever quicker, the picture forms. For instance: There would seem to be no connection between the 'Covid pandemic' hoax and the human-caused global-warming hoax and yet they are masks (appropriately) on the same face seeking the same outcome. Those pushing the global warming myth through the Club of Rome and other Cult agencies are driving the lies about 'Covid' – Bill Gates is an obvious one, but they are endless. Why would the same people be involved in both when they are clearly not connected? Oh, but they

are. Common themes with personnel are matched by common goals. The 'solutions' to both 'problems' are centralisation of global power to impose the will of the few on the many to 'save' humanity from 'Covid' and save the planet from an 'existential threat' (we need 'zero Covid' and 'zero carbon emissions'). These, in turn, connect with the 'dot' of globalisation which was coined to describe the centralisation of global power in every area of life through incessant political and corporate expansion, trading blocks and superstates like the European Union. If you are the few and you want to control the many you have to centralise power and decision-making. The more you centralise power the more power the few at the centre will have over the many; and the more that power is centralised the more power those at the centre have to centralise even quicker. The momentum of centralisation gets faster and faster which is exactly the process we have witnessed. In this way the hoaxed 'pandemic' and the fakery of human-caused global warming serve the interests of globalisation and the seizure of global power in the hands of the Cult inner-circle which is behind 'Covid', 'climate change' *and* globalisation. At this point random 'dots' become a clear and obvious picture or pattern.

Klaus Schwab, the classic Bond villain who founded the Cult's Gates-funded World Economic Forum, published a book in 2020, *The Great Reset*, in which he used the 'problem' of 'Covid' to justify a total transformation of human society to 'save' humanity from 'climate change'. Schwab said: 'The pandemic represents a rare but narrow window of opportunity to reflect, reimagine, and reset our world.' What he didn't mention is that the Cult he serves is behind both hoaxes as I show in my book *The Answer*. He and the Cult don't have to reimagine the world. They know precisely what they want and that's why they destroyed human society with 'Covid' to 'build back better' in their grand design. Their job is not to imagine, but to get humanity to imagine and agree with their plans while believing it's all random. It must be pure coincidence that 'The Great Reset' has long been the Cult's code name for the global imposition of fascism and replaced previous code-names of the 'New World

Order' used by Cult frontmen like Father George Bush and the 'New Order of the Ages' which emerged from Freemasonry and much older secret societies. New Order of the Ages appears on the reverse of the Great Seal of the United States as 'Novus ordo seclorum' underneath the Cult symbol used since way back of the pyramid and all seeing-eye (Fig 3). The pyramid is the hierarchy of human control headed by the illuminated eye that symbolises the force behind the Cult which I will expose in later chapters. The term 'Annuit Coeptis' translates as 'He favours our undertaking'. We are told the 'He' is the Christian god, but 'He' is not as I will be explaining.

Figure 3: The all-seeing eye of the Cult 'god' on the Freemason-designed Great Seal of the United States and also on the dollar bill.

Having you on

Two major Cult techniques of perceptual manipulation that relate to all this are what I have called since the 1990s Problem-Reaction-Solution (PRS) and the Totalitarian Tiptoe (TT). They can be uncovered by the inquiring mind with a simple question: Who benefits? The answer usually identifies the perpetrators of a given action or happening through the concept of 'he who most benefits from a crime is the one most likely to have committed it'. The Latin 'Cue bono?' – Who benefits? – is widely attributed to the Roman orator and statesman Marcus Tullius Cicero. No wonder it goes back so far when the concept has been relevant to human behaviour since

history was recorded. Problem-Reaction-Solution is the technique used to manipulate us every day by covertly creating a problem (or the illusion of one) and offering the solution to the problem (or the illusion of one). In the first phase you create the problem and blame someone or something else for why it has happened. This may relate to a financial collapse, terrorist attack, war, global warming or pandemic, anything in fact that will allow you to impose the 'solution' to change society in the way you desire at that time. The 'problem' doesn't have to be real. PRS is manipulation of perception and all you need is the population to believe the problem is real. Human-caused global warming and the 'Covid pandemic' only have to be *perceived* to be real for the population to accept the 'solutions' of authority. I refer to this technique as NO-Problem-Reaction-Solution. Billions did not meekly accept house arrest from early 2020 because there was a real deadly 'Covid pandemic' but because they perceived – believed – that to be the case. The antidote to Problem-Reaction-Solution is to ask who benefits from the proposed solution. Invariably it will be anyone who wants to justify more control through deletion of freedom and centralisation of power and decision-making.

The two world wars were Problem-Reaction-Solutions that transformed and realigned global society. Both were manipulated into being by the Cult as I have detailed in books since the mid-1990s. They dramatically centralised global power, especially World War Two, which led to the United Nations and other global bodies thanks to the overt and covert manipulations of the Rockefeller family and other Cult bloodlines like the Rothschilds. The UN is a stalking horse for full-blown world government that I will come to shortly. The land on which the UN building stands in New York was donated by the Rockefellers and the same Cult family was behind Big Pharma scalpel and drug 'medicine' and the creation of the World Health Organization as part of the UN. They have been stalwarts of the eugenics movement and funded Hitler's race-purity expert' Ernst Rudin. The human-caused global warming hoax has been orchestrated by the Club of Rome through the UN which is

manufacturing both the 'problem' through its Intergovernmental Panel on Climate Change and imposing the 'solution' through its Agenda 21 and Agenda 2030 which demand the total centralisation of global power to 'save the world' from a climate hoax the United Nations is itself perpetrating. What a small world the Cult can be seen to be particularly among the inner circles. The bedfellow of Problem-Reaction-Solution is the Totalitarian Tiptoe which became the Totalitarian Sprint in 2020. The technique is fashioned to hide the carefully-coordinated behind the cover of apparently random events. You start the sequence at 'A' and you know you are heading for 'Z'. You don't want people to know that and each step on the journey is presented as a random happening while all the steps strung together lead in the same direction. The speed may have quickened dramatically in recent times, but you can still see the incremental approach of the Tiptoe in the case of 'Covid' as each new imposition takes us deeper into fascism. Tell people they have to do this or that to get back to 'normal', then this and this and this. With each new demand adding to the ones that went before the population's freedom is deleted until it disappears. The spider wraps its web around the flies more comprehensively with each new diktat. I'll highlight this in more detail when I get to the 'Covid' hoax and how it has been pulled off. Another prime example of the Totalitarian Tiptoe is how the Cult-created European Union went from a 'free-trade zone' to a centralised bureaucratic dictatorship through the Tiptoe of incremental centralisation of power until nations became mere administrative units for Cult-owned dark suits in Brussels.

The antidote to ignorance is knowledge which the Cult seeks vehemently to deny us, but despite the systematic censorship to that end the Renegade Mind can overcome this by vociferously seeking out the facts no matter the impediments put in the way. There is also a method of thinking and perceiving – *knowing* – that doesn't even need names, dates, place-type facts to identify the patterns that reveal the story. I'll get to that in the final chapter. All you need to know about the manipulation of human society and to what end is still out there – *at the time of writing* – in the form of books, videos

and websites for those that really want to breach the walls of programmed perception. To access this knowledge requires the abandonment of the mainstream media as a source of information in the awareness that this is owned and controlled by the Cult and therefore promotes mass perceptions that suit the Cult. Mainstream media lies all day, every day. That is its function and very reason for being. Where it does tell the truth, here and there, is only because the truth and the Cult agenda very occasionally coincide. If you look for fact and insight to the BBC, CNN and virtually all the rest of them you are asking to be conned and perceptually programmed.

Know the outcome and you'll see the journey

Events seem random when you have no idea where the world is being taken. Once you do the random becomes the carefully planned. Know the outcome and you'll see the journey is a phrase I have been using for a long time to give context to daily happenings that appear unconnected. Does a problem, or illusion of a problem, trigger a proposed 'solution' that further drives society in the direction of the outcome? Invariably the answer will be yes and the random – *abracadabra* – becomes the clearly coordinated. So what is this outcome that unlocks the door to a massively expanded understanding of daily events? I will summarise its major aspects – the fine detail is in my other books – and those new to this information will see that the world they thought they were living in is a very different place. The foundation of the Cult agenda is the incessant centralisation of power and all such centralisation is ultimately in pursuit of Cult control on a global level. I have described for a long time the planned world structure of top-down dictatorship as the Hunger Games Society. The term obviously comes from the movie series which portrayed a world in which a few living in military-protected hi-tech luxury were the overlords of a population condemned to abject poverty in isolated 'sectors' that were not allowed to interact. 'Covid' lockdowns and travel bans anyone? The 'Hunger Games' pyramid of structural control has the inner circle of the Cult at the top with pretty much the entire

population at the bottom under their control through dependency for survival on the Cult. The whole structure is planned to be protected and enforced by a military-police state (Fig 4).

Here you have the reason for the global lockdowns of the fake pandemic to coldly destroy independent incomes and livelihoods and make everyone dependent on the 'state' (the Cult that controls the 'states'). I have warned in my books for many years about the plan to introduce a 'guaranteed income' – a barely survivable pittance – designed to impose dependency when employment was destroyed by AI technology and now even more comprehensively at great speed by the 'Covid' scam. Once the pandemic was played and lockdown consequences began to delete independent income the authorities began to talk right on cue about the need for a guaranteed income and a 'Great Reset'. Guaranteed income will be presented as benevolent governments seeking to help a desperate people – desperate as a direct result of actions of the same governments. The truth is that such payments are a trap. You will only get them if you do exactly what the authorities demand including mass vaccination (genetic manipulation). We have seen this theme already in Australia where those dependent on government benefits have them reduced if parents don't agree to have their children vaccinated according to an insane health-destroying government-dictated schedule. Calculated economic collapse applies to governments as well as people. The Cult wants rid of countries through the creation of a world state with countries broken up into regions ruled by a world government and super states like the European Union. Countries must be bankrupted, too, to this end and it's being achieved by the trillions in 'rescue packages' and furlough payments, trillions in lost taxation, and money-no-object spending on 'Covid' including constant all-medium advertising (programming) which has made the media dependent on government for much of its income. The day of reckoning is coming – as planned – for government spending and given that it has been made possible by printing money and not by production/taxation there is inflation on the way that has the

potential to wipe out monetary value. In that case there will be no need for the Cult to steal your money. It just won't be worth anything (see the German Weimar Republic before the Nazis took over). Many have been okay with lockdowns while getting a percentage of their income from so-called furlough payments without having to work. Those payments are dependent, however, on people having at least a theoretical job with a business considered non-essential and ordered to close. As these business go under because they are closed by lockdown after lockdown the furlough stops and it will for everyone eventually. Then what? The 'then what?' is precisely the idea.

THE COVID-19 CREATED HUNGER GAMES SOCIETY
THE ELITE - 1%
THE GLOBAL POLICE STATE
MANDATORY VACCINATION - ID - SOCIAL CREDIT - 666
CLOSED
FOOD BANK
THE MASSES LIVING IN POVERTY

Figure 4: The Hunger Games Society structure I have long warned was planned and now the 'Covid' hoax has made it possible. This is the real reason for lockdowns.

Hired hands

Between the Hunger Games Cult elite and the dependent population is planned to be a vicious military-police state (a fusion of the two into one force). This has been in the making for a long time with police looking ever more like the military and carrying weapons to match. The pandemic scam has seen this process accelerate so fast as

lockdown house arrest is brutally enforced by carefully recruited fascist minds and gormless system-servers. The police and military are planned to merge into a centrally-directed world army in a global structure headed by a world government which wouldn't be elected even by the election fixes now in place. The world army is not planned even to be human and instead wars would be fought, primarily against the population, using robot technology controlled by artificial intelligence. I have been warning about this for decades and now militaries around the world are being transformed by this very AI technology. The global regime that I describe is a particular form of fascism known as a technocracy in which decisions are not made by clueless and co-opted politicians but by unelected technocrats – scientists, engineers, technologists and bureaucrats. Cult-owned-and-controlled Silicon Valley giants are examples of technocracy and they already have far more power to direct world events than governments. They are with their censorship *selecting* governments. I know that some are calling the 'Great Reset' a Marxist communist takeover, but fascism and Marxism are different labels for the same tyranny. Tell those who lived in fascist Germany and Stalinist Russia that there was a difference in the way their freedom was deleted and their lives controlled. I could call it a fascist technocracy or a Marxist technocracy and they would be equally accurate. The Hunger Games society with its world government structure would oversee a world army, world central bank and single world cashless currency imposing its will on a microchipped population (Fig 5). Scan its different elements and see how the illusory pandemic is forcing society in this very direction at great speed. Leaders of 23 countries and the World Health Organization (WHO) backed the idea in March, 2021, of a global treaty for 'international cooperation' in 'health emergencies' and nations should 'come together as a global community for peaceful cooperation that extends beyond this crisis'. Cut the Orwellian bullshit and this means another step towards global government. The plan includes a cashless digital money system that I first warned about in 1993. Right at the start of 'Covid' the deeply corrupt Tedros

Adhanom Ghebreyesus, the crooked and merely gofer 'head' of the World Health Organization, said it was possible to catch the 'virus' by touching cash and it was better to use cashless means. The claim was ridiculous nonsense and like the whole 'Covid' mind-trick it was nothing to do with 'health' and everything to do with pushing every aspect of the Cult agenda. As a result of the Tedros lie the use of cash has plummeted. The Cult script involves a single world digital currency that would eventually be technologically embedded in the body. China is a massive global centre for the Cult and if you watch what is happening there you will know what is planned for everywhere. The Chinese government is developing a digital currency which would allow fines to be deducted immediately via AI for anyone caught on camera breaking its fantastic list of laws and the money is going to be programmable with an expiry date to ensure that no one can accrue wealth except the Cult and its operatives.

WORLD GOVERNMENT
UNELECTED - RUN BY TECHNOCRATS
WORLD ARMY • CENTRAL BANK • CURRENCY •
HUMANS CONNECTED TO A.I.
SUPERSTATES LIKE THE EU AND CENTRALLY
CONTROLLED 'TRADING BLOCS'
COUNTRIES REPLACED BY POWERLESS REGIONS
GLOBAL MONOCULTURE
THE PEOPLE

Figure 5: The structure of global control the Cult has been working towards for so long and this has been enormously advanced by the 'Covid' illusion.

Serfdom is so smart

The Cult plan is far wider, extreme, and more comprehensive than even most conspiracy researchers appreciate and I will come to the true depths of deceit and control in the chapters 'Who controls the

Cult?' and 'Escaping Wetiko'. Even the world that we know is crazy enough. We are being deluged with ever more sophisticated and controlling technology under the heading of 'smart'. We have smart televisions, smart meters, smart cards, smart cars, smart driving, smart roads, smart pills, smart patches, smart watches, smart skin, smart borders, smart pavements, smart streets, smart cities, smart communities, smart environments, smart growth, smart planet ... smart *everything* around us. Smart technologies and methods of operation are designed to interlock to create a global Smart Grid connecting the entirety of human society including human minds to create a centrally-dictated 'hive' mind. 'Smart cities' is code for densely-occupied megacities of total surveillance and control through AI. Ever more destructive frequency communication systems like 5G have been rolled out without any official testing for health and psychological effects (colossal). 5G/6G/7G systems are needed to run the Smart Grid and each one becomes more destructive of body and mind. Deleting independent income is crucial to forcing people into these AI-policed prisons by ending private property ownership (except for the Cult elite). The Cult's Great Reset now openly foresees a global society in which no one will own any possessions and everything will be rented while the Cult would own literally everything under the guise of government and corporations. The aim has been to use the lockdowns to destroy sources of income on a mass scale and when the people are destitute and in unrepayable amounts of debt (problem) Cult assets come forward with the pledge to write-off debt in return for handing over all property and possessions (solution). Everything – literally everything including people – would be connected to the Internet via AI. I was warning years ago about the coming Internet of Things (IoT) in which all devices and technology from your car to your fridge would be plugged into the Internet and controlled by AI. Now we are already there with much more to come. The next stage is the Internet of Everything (IoE) which is planned to include the connection of AI to the human brain and body to replace the human mind with a centrally-controlled AI mind. Instead of perceptions

being manipulated through control of information and censorship those perceptions would come direct from the Cult through AI. What do you think? You think whatever AI decides that you think. In human terms there would be no individual 'think' any longer. Too incredible? The ravings of a lunatic? Not at all. Cult-owned crazies in Silicon Valley have been telling us the plan for years without explaining the real motivation and calculated implications. These include Google executive and 'futurist' Ray Kurzweil who highlights the year 2030 for when this would be underway. He said:

Our thinking ... will be a hybrid of biological and non-biological thinking ... humans will be able to extend their limitations and 'think in the cloud' ... We're going to put gateways to the cloud in our brains ... We're going to gradually merge and enhance ourselves ... In my view, that's the nature of being human – we transcend our limitations.

As the technology becomes vastly superior to what we are then the small proportion that is still human gets smaller and smaller and smaller until it's just utterly negligible.

The sales-pitch of Kurzweil and Cult-owned Silicon Valley is that this would make us 'super-human' when the real aim is to make us post-human and no longer 'human' in the sense that we have come to know. The entire global population would be connected to AI and become the centrally-controlled 'hive-mind' of externally-delivered perceptions. The Smart Grid being installed to impose the Cult's will on the world is being constructed to allow particular locations – even one location – to control the whole global system. From these prime control centres, which absolutely include China and Israel, anything connected to the Internet would be switched on or off and manipulated at will. Energy systems could be cut, communication via the Internet taken down, computer-controlled driverless autonomous vehicles driven off the road, medical devices switched off, the potential is limitless given how much AI and Internet connections now run human society. We have seen nothing yet if we allow this to continue. Autonomous vehicle makers are working with law enforcement to produce cars designed to automatically pull over if they detect a police or emergency vehicle flashing from up to 100 feet away. At a police stop the car would be unlocked and the

window rolled down automatically. Vehicles would only take you where the computer (the state) allowed. The end of petrol vehicles and speed limiters on all new cars in the UK and EU from 2022 are steps leading to electric computerised transport over which ultimately you have no control. The picture is far bigger even than the Cult global network or web and that will become clear when I get to the nature of the 'spider'. There is a connection between all these happenings and the instigation of DNA-manipulating 'vaccines' (which aren't 'vaccines') justified by the 'Covid' hoax. That connection is the unfolding plan to transform the human body from a biological to a synthetic biological state and this is why synthetic biology is such a fast-emerging discipline of mainstream science. 'Covid vaccines' are infusing self-replicating synthetic genetic material into the cells to cumulatively take us on the Totalitarian Tiptoe from Human 1.0 to the synthetic biological Human 2.0 which will be physically and perceptually attached to the Smart Grid to one hundred percent control every thought, perception and deed. Humanity needs to wake up and *fast*.

This is the barest explanation of where the 'outcome' is planned to go but it's enough to see the journey happening all around us. Those new to this information will already see 'Covid' in a whole new context. I will add much more detail as we go along, but for the minutiae evidence see my mega-works, *The Answer, The Trigger* and *Everything You Need to Know But Have Never Been Told*.

Now – how does a Renegade Mind see the 'world'?

CHAPTER TWO

Renegade Perception

It is one thing to be clever and another to be wise
George R.R. Martin

A simple definition of the difference between a programmed mind and a Renegade Mind would be that one sees only dots while the other connects them to see the picture. Reading reality with accuracy requires the observer to (a) know the planned outcome and (b) realise that everything, but *everything*, is connected.

The entirety of infinite reality is connected – that's its very nature – and with human society an expression of infinite reality the same must apply. Simple cause and effect is a connection. The effect is triggered by the cause and the effect then becomes the cause of another effect. Nothing happens in isolation because it *can't*. Life in whatever reality is simple choice and consequence. We make choices and these lead to consequences. If we don't like the consequences we can make different choices and get different consequences which lead to other choices and consequences. The choice and the consequence are not only connected they are indivisible. You can't have one without the other as an old song goes. A few cannot control the world unless those being controlled allow that to happen – cause and effect, choice and consequence. Control – who has it and who doesn't – is a two-way process, a symbiotic relationship, involving the controller and controlled. 'They took my freedom away!!' Well, yes, but you also gave it to them. Humanity is

subjected to mass control because humanity has acquiesced to that control. This is all cause and effect and literally a case of give and take. In the same way world events of every kind are connected and the Cult works incessantly to sell the illusion of the random and coincidental to maintain the essential (to them) perception of dots that hide the picture. Renegade Minds know this and constantly scan the world for patterns of connection. This is absolutely pivotal in understanding the happenings in the world and without that perspective clarity is impossible. First you know the planned outcome and then you identify the steps on the journey – the day-by-day apparently random which, when connected in relation to the outcome, no longer appear as individual events, but as the proverbial *chain* of events leading in the same direction. I'll give you some examples:

Political puppet show

We are told to believe that politics is 'adversarial' in that different parties with different beliefs engage in an endless tussle for power. There may have been some truth in that up to a point – and only a point – but today divisions between 'different' parties are rhetorical not ideological. Even the rhetorical is fusing into one-speak as the parties eject any remaining free thinkers while others succumb to the ever-gathering intimidation of anyone with the 'wrong' opinion. The Cult is not a new phenomenon and can be traced back thousands of years as my books have documented. Its intergenerational initiates have been manipulating events with increasing effect the more that global power has been centralised. In ancient times the Cult secured control through the system of monarchy in which 'special' bloodlines (of which more later) demanded the right to rule as kings and queens simply by birthright and by vanquishing others who claimed the same birthright. There came a time, however, when people had matured enough to see the unfairness of such tyranny and demanded a say in who governed them. Note the word – *governed* them. Not served them – *governed* them, hence government defined as 'the political direction and control exercised over the

actions of the members, citizens, or inhabitants of communities, societies, and states; direction of the affairs of a state, community, etc.' Governments exercise control over rather than serve just like the monarchies before them. Bizarrely there are still countries like the United Kingdom which are ruled by a monarch *and* a government that officially answers to the monarch. The UK head of state and that of Commonwealth countries such as Canada, Australia and New Zealand is 'selected' by who in a *single family* had unprotected sex with whom and in what order. Pinch me it can't be true. Ouch! Shit, it is. The demise of monarchies in most countries offered a potential vacuum in which some form of free and fair society could arise and the Cult had that base covered. Monarchies had served its interests but they couldn't continue in the face of such widespread opposition and, anyway, replacing a 'royal' dictatorship that people could see with a dictatorship 'of the people' hiding behind the concept of 'democracy' presented far greater manipulative possibilities and ways of hiding coordinated tyranny behind the illusion of 'freedom'.

Democracy is quite wrongly defined as government selected by the population. This is not the case at all. It is government selected by *some* of the population (and then only in theory). This 'some' doesn't even have to be the majority as we have seen so often in first-past-the-post elections in which the so-called majority party wins fewer votes than the 'losing' parties combined. Democracy can give total power to a party in government from a minority of the votes cast. It's a sleight of hand to sell tyranny as freedom. Seventy-four million Trump-supporting Americans didn't vote for the 'Democratic' Party of Joe Biden in the distinctly dodgy election in 2020 and yet far from acknowledging the wishes and feelings of that great percentage of American society the Cult-owned Biden government set out from day one to destroy them and their right to a voice and opinion. Empty shell Biden and his Cult handlers said they were doing this to 'protect democracy'. Such is the level of lunacy and sickness to which politics has descended. Connect the dots and relate them to the desired outcome – a world government run by self-appointed technocrats and no longer even elected

politicians. While operating through its political agents in government the Cult is at the same time encouraging public distain for politicians by putting idiots and incompetents in theoretical power on the road to deleting them. The idea is to instil a public reaction that says of the technocrats: 'Well, they couldn't do any worse than the pathetic politicians.' It's all about controlling perception and Renegade Minds can see through that while programmed minds cannot when they are ignorant of both the planned outcome and the manipulation techniques employed to secure that end. This knowledge can be learned, however, and fast if people choose to get informed.

Politics may at first sight appear very difficult to control from a central point. I mean look at the 'different' parties and how would you be able to oversee them all and their constituent parts? In truth, it's very straightforward because of their structure. We are back to the pyramid of imposition and acquiescence. Organisations are structured in the same way as the system as a whole. Political parties are not open forums of free expression. They are hierarchies. I was a national spokesman for the British Green Party which claimed to be a different kind of politics in which influence and power was devolved; but I can tell you from direct experience – and it's far worse now – that Green parties are run as hierarchies like all the others however much they may try to hide that fact or kid themselves that it's not true. A very few at the top of all political parties are directing policy and personnel. They decide if you are elevated in the party or serve as a government minister and to do that you have to be a yes man or woman. Look at all the maverick political thinkers who never ascended the greasy pole. If you want to progress within the party or reach 'high-office' you need to fall into line and conform. Exceptions to this are rare indeed. Should you want to run for parliament or Congress you have to persuade the local or state level of the party to select you and for that you need to play the game as dictated by the hierarchy. If you secure election and wish to progress within the greater structure you need to go on conforming to what is acceptable to those running the hierarchy

from the peak of the pyramid. Political parties are perceptual gulags and the very fact that there are party 'Whips' appointed to 'whip' politicians into voting the way the hierarchy demands exposes the ridiculous idea that politicians are elected to serve the people they are supposed to represent. Cult operatives and manipulation has long seized control of major parties that have any chance of forming a government and at least most of those that haven't. A new party forms and the Cult goes to work to infiltrate and direct. This has reached such a level today that you see video compilations of 'leaders' of all parties whether Democrats, Republicans, Conservative, Labour and Green parroting the same Cult mantra of 'Build Back Better' and the 'Great Reset' which are straight off the Cult song-sheet to describe the transformation of global society in response to the Cult-instigated hoaxes of the 'Covid pandemic' and human-caused 'climate change'. To see Caroline Lucas, the Green Party MP that I knew when I was in the party in the 1980s, speaking in support of plans proposed by Cult operative Klaus Schwab representing the billionaire global elite is a real head-shaker.

Many parties – one master

The party system is another mind-trick and was instigated to change the nature of the dictatorship by swapping 'royalty' for dark suits that people believed – though now ever less so – represented their interests. Understanding this trick is to realise that a single force (the Cult) controls all parties either directly in terms of the major ones or through manipulation of perception and ideology with others. You don't need to manipulate Green parties to demand your transformation of society in the name of 'climate change' when they are obsessed with the lie that this is essential to 'save the planet'. You just give them a platform and away they go serving your interests while believing they are being environmentally virtuous. America's political structure is a perfect blueprint for how the two or multi-party system is really a one-party state. The Republican Party is controlled from one step back in the shadows by a group made up of billionaires and their gofers known as neoconservatives or Neocons.

I have exposed them in fine detail in my books and they were the driving force behind the policies of the imbecilic presidency of Boy George Bush which included 9/11 (see *The Trigger* for a comprehensive demolition of the official story), the subsequent 'war on terror' (war *of* terror) and the invasions of Afghanistan and Iraq. The latter was a No-Problem-Reaction-Solution based on claims by Cult operatives, including Bush and British Prime Minister Tony Blair, about Saddam Hussein's 'weapons of mass destruction' which did not exist as war criminals Bush and Blair well knew.

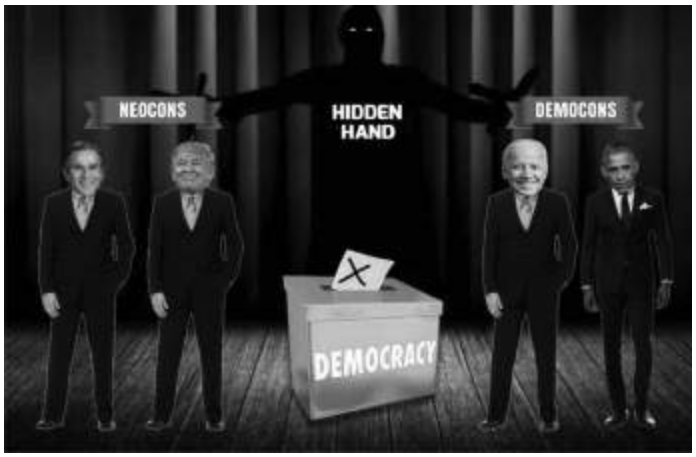

Figure 6: Different front people, different parties – same control system.

The Democratic Party has its own 'Neocon' group controlling from the background which I call the 'Democons' and here's the penny-drop – the Neocons and Democons answer to the same masters one step further back into the shadows (Fig 6). At that level of the Cult the Republican and Democrat parties are controlled by the same people and no matter which is in power the Cult is in power. This is how it works in almost every country and certainly in Britain with Conservative, Labour, Liberal Democrat and Green parties now all on the same page whatever the rhetoric may be in their feeble attempts to appear different. Neocons operated at the time of Bush through a think tank called The Project for the New American Century which in September, 2000, published a document entitled *Rebuilding America's Defenses: Strategies, Forces, and Resources*

For a New Century demanding that America fight 'multiple, simultaneous major theatre wars' as a 'core mission' to force regime-change in countries including Iraq, Libya and Syria. Neocons arranged for Bush ('Republican') and Blair ('Labour Party') to front-up the invasion of Iraq and when they departed the Democons orchestrated the targeting of Libya and Syria through Barack Obama ('Democrat') and British Prime Minister David Cameron ('Conservative Party'). We have 'different' parties and 'different' people, but the same unfolding script. The more the Cult has seized the reigns of parties and personnel the more their policies have transparently pursued the same agenda to the point where the fascist 'Covid' impositions of the Conservative junta of Jackboot Johnson in Britain were opposed by the Labour Party because they were not fascist enough. The Labour Party is likened to the US Democrats while the Conservative Party is akin to a British version of the Republicans and on both sides of the Atlantic they all speak the same language and support the direction demanded by the Cult although some more enthusiastically than others. It's a similar story in country after country because it's all centrally controlled. Oh, but what about Trump? I'll come to him shortly. Political 'choice' in the 'party' system goes like this: You vote for Party A and they get into government. You don't like what they do so next time you vote for Party B and they get into government. You don't like what they do when it's pretty much the same as Party A and why wouldn't that be with both controlled by the same force? Given that only two, sometimes three, parties have any chance of forming a government to get rid of Party B that you don't like you have to vote again for Party A which … you don't like. This, ladies and gentlemen, is what they call 'democracy' which we are told – wrongly – is a term interchangeable with 'freedom'.

The cult of cults

At this point I need to introduce a major expression of the Global Cult known as Sabbatian-Frankism. Sabbatian is also spelt as Sabbatean. I will summarise here. I have published major exposés

and detailed background in other works. Sabbatian-Frankism combines the names of two frauds posing as 'Jewish' men, Sabbatai Zevi (1626-1676), a rabbi, black magician and occultist who proclaimed he was the Jewish messiah; and Jacob Frank (1726-1791), the Polish 'Jew', black magician and occultist who said he was the reincarnation of 'messiah' Zevi and biblical patriarch Jacob. They worked across two centuries to establish the Sabbatian-Frankist cult that plays a major, indeed central, role in the manipulation of human society by the Global Cult which has its origins much further back in history than Sabbatai Zevi. I should emphasise two points here in response to the shrill voices that will scream 'anti-Semitism': (1) Sabbatian-Frankists are NOT Jewish and only pose as such to hide their cult behind a Jewish façade; and (2) my information about this cult has come from Jewish sources who have long realised that their society and community has been infiltrated and taken over by interloper Sabbatian-Frankists. Infiltration has been the foundation technique of Sabbatian-Frankism from its official origin in the 17th century. Zevi's Sabbatian sect attracted a massive following described as the biggest messianic movement in Jewish history, spreading as far as Africa and Asia, and he promised a return for the Jews to the 'Promised Land' of Israel. Sabbatianism was not Judaism but an inversion of everything that mainstream Judaism stood for. So much so that this sinister cult would have a feast day when Judaism had a fast day and whatever was forbidden in Judaism the Sabbatians were encouraged and even commanded to do. This included incest and what would be today called Satanism. Members were forbidden to marry outside the sect and there was a system of keeping their children ignorant of what they were part of until they were old enough to be trusted not to unknowingly reveal anything to outsiders. The same system is employed to this day by the Global Cult in general which Sabbatian-Frankism has enormously influenced and now largely controls.

Zevi and his Sabbatians suffered a setback with the intervention by the Sultan of the Islamic Ottoman Empire in the Middle East and what is now the Republic of Turkey where Zevi was located. The

Sultan gave him the choice of proving his 'divinity', converting to Islam or facing torture and death. Funnily enough Zevi chose to convert or at least appear to. Some of his supporters were disillusioned and drifted away, but many did not with 300 families also converting – only in theory – to Islam. They continued behind this Islamic smokescreen to follow the goals, rules and rituals of Sabbatianism and became known as 'crypto-Jews' or the 'Dönmeh' which means 'to turn'. This is rather ironic because they didn't 'turn' and instead hid behind a fake Islamic persona. The process of appearing to be one thing while being very much another would become the calling card of Sabbatianism especially after Zevi's death and the arrival of the Satanist Jacob Frank in the 18th century when the cult became Sabbatian-Frankism and plumbed still new depths of depravity and infiltration which included – still includes – human sacrifice and sex with children. Wherever Sabbatians go paedophilia and Satanism follow and is it really a surprise that Hollywood is so infested with child abuse and Satanism when it was established by Sabbatian-Frankists and is still controlled by them? Hollywood has been one of the prime vehicles for global perceptual programming and manipulation. How many believe the version of 'history' portrayed in movies when it is a travesty and inversion (again) of the truth? Rabbi Marvin Antelman describes Frankism in his book, *To Eliminate the Opiate*, as 'a movement of complete evil' while Jewish professor Gershom Scholem said of Frank in *The Messianic Idea in Judaism*: 'In all his actions [he was] a truly corrupt and degenerate individual ... one of the most frightening phenomena in the whole of Jewish history.' Frank was excommunicated by traditional rabbis, as was Zevi, but Frank was undeterred and enjoyed vital support from the House of Rothschild, the infamous banking dynasty whose inner-core are Sabbatian-Frankists and not Jews. Infiltration of the Roman Church and Vatican was instigated by Frank with many Dönmeh 'turning' again to convert to Roman Catholicism with a view to hijacking the reins of power. This was the ever-repeating modus operandi and continues to be so. Pose as an advocate of the religion, culture or country that you want to control and then

manipulate your people into the positions of authority and influence largely as advisers, administrators and Svengalis for those that appear to be in power. They did this with Judaism, Christianity (Christian Zionism is part of this), Islam and other religions and nations until Sabbatian-Frankism spanned the world as it does today.

Sabbatian Saudis and the terror network

One expression of the Sabbatian-Frankist Dönmeh within Islam is the ruling family of Saudi Arabia, the House of Saud, through which came the vile distortion of Islam known as Wahhabism. This is the violent creed followed by terrorist groups like Al-Qaeda and ISIS or Islamic State. Wahhabism is the hand-chopping, head-chopping 'religion' of Saudi Arabia which is used to keep the people in a constant state of fear so the interloper House of Saud can continue to rule. Al-Qaeda and Islamic State were lavishly funded by the House of Saud while being created and directed by the Sabbatian-Frankist network in the United States that operates through the Pentagon, CIA and the government in general of whichever 'party'. The front man for the establishment of Wahhabism in the middle of the 18th century was a Sabbatian-Frankist 'crypto-Jew' posing as Islamic called Muhammad ibn Abd al-Wahhab. His daughter would marry the son of Muhammad bin Saud who established the first Saudi state before his death in 1765 with support from the British Empire. Bin Saud's successors would establish modern Saudi Arabia in league with the British and Americans in 1932 which allowed them to seize control of Islam's major shrines in Mecca and Medina. They have dictated the direction of Sunni Islam ever since while Iran is the major centre of the Shiite version and here we have the source of at least the public conflict between them. The Sabbatian network has used its Wahhabi extremists to carry out Problem-Reaction-Solution terrorist attacks in the name of 'Al-Qaeda' and 'Islamic State' to justify a devastating 'war on terror', ever-increasing surveillance of the population and to terrify people into compliance. Another insight of the Renegade Mind is the streetwise understanding that

just because a country, location or people are attacked doesn't mean that those apparently representing that country, location or people are not behind the attackers. Often they are *orchestrating* the attacks because of the societal changes that can be then justified in the name of 'saving the population from terrorists'.

I show in great detail in *The Trigger* how Sabbatian-Frankists were the real perpetrators of 9/11 and not '19 Arab hijackers' who were blamed for what happened. Observe what was justified in the name of 9/11 alone in terms of Middle East invasions, mass surveillance and control that fulfilled the demands of the Project for the New American Century document published by the Sabbatian Neocons. What appear to be enemies are on the deep inside players on the same Sabbatian team. Israel and Arab 'royal' dictatorships are all ruled by Sabbatians and the recent peace agreements between Israel and Saudi Arabia, the United Arab Emirates (UAE) and others are only making formal what has always been the case behind the scenes. Palestinians who have been subjected to grotesque tyranny since Israel was bombed and terrorised into existence in 1948 have never stood a chance. Sabbatian-Frankists have controlled Israel (so the constant theme of violence and war which Sabbatians love) and they have controlled the Arab countries that Palestinians have looked to for real support that never comes. 'Royal families' of the Arab world in Saudi Arabia, Bahrain, UAE, etc., are all Sabbatians with allegiance to the aims of the cult and not what is best for their Arabic populations. They have stolen the oil and financial resources from their people by false claims to be 'royal dynasties' with a genetic right to rule and by employing vicious militaries to impose their will.

Satanic 'illumination'

The Satanist Jacob Frank formed an alliance in 1773 with two other Sabbatians, Mayer Amschel Rothschild (1744-1812), founder of the Rothschild banking dynasty, and Jesuit-educated fraudulent Jew, Adam Weishaupt, and this led to the formation of the Bavarian Illuminati, firstly under another name, in 1776. The Illuminati would

be the manipulating force behind the French Revolution (1789-1799) and was also involved in the American Revolution (1775-1783) before and after the Illuminati's official creation. Weishaupt would later become (in public) a Protestant Christian in archetypal Sabbatian style. I read that his name can be decoded as Adam-Weishaupt or 'the first man to lead those who know'. He wasn't a leader in the sense that he was a subordinate, but he did lead those below him in a crusade of transforming human society that still continues today. The theme was confirmed as early as 1785 when a horseman courier called Lanz was reported to be struck by lighting and extensive Illuminati documents were found in his saddlebags. They made the link to Weishaupt and detailed the plan for world takeover. Current events with 'Covid' fascism have been in the making for a very long time. Jacob Frank was jailed for 13 years by the Catholic Inquisition after his arrest in 1760 and on his release he headed for Frankfurt, Germany, home city and headquarters of the House of Rothschild where the alliance was struck with Mayer Amschel Rothschild and Weishaupt. Rothschild arranged for Frank to be given the title of Baron and he became a wealthy nobleman with a big following of Jews in Germany, the Austro-Hungarian Empire and other European countries. Most of them would have believed he was on their side.

The name 'Illuminati' came from the Zohar which is a body of works in the Jewish mystical 'bible' called the Kabbalah. 'Zohar' is the foundation of Sabbatian-Frankist belief and in Hebrew 'Zohar' means 'splendour', 'radiance', 'illuminated', and so we have 'Illuminati'. They claim to be the 'Illuminated Ones' from their knowledge systematically hidden from the human population and passed on through generations of carefully-chosen initiates in the global secret society network or Cult. Hidden knowledge includes an awareness of the Cult agenda for the world and the nature of our collective reality that I will explore later. Cult 'illumination' is symbolised by the torch held by the Statue of Liberty which was gifted to New York by French Freemasons in Paris who knew exactly what it represents. 'Liberty' symbolises the goddess worshipped in

Babylon as Queen Semiramis or Ishtar. The significance of this will become clear. Notice again the ubiquitous theme of inversion with the Statue of 'Liberty' really symbolising mass control (Fig 7). A mirror-image statute stands on an island in the River Seine in Paris from where New York Liberty originated (Fig 8). A large replica of the Liberty flame stands on top of the Pont de l'Alma tunnel in Paris where Princess Diana died in a Cult ritual described in *The Biggest Secret*. Lucifer 'the light bringer' is related to all this (and much more as we'll see) and 'Lucifer' is a central figure in Sabbatian-Frankism and its associated Satanism. Sabbatians reject the Jewish Torah, or Pentateuch, the 'five books of Moses' in the Old Testament known as Genesis, Exodus, Leviticus, Numbers, and Deuteronomy which are claimed by Judaism and Christianity to have been dictated by 'God' to Moses on Mount Sinai. Sabbatians say these do not apply to them and they seek to replace them with the Zohar to absorb Judaism and its followers into their inversion which is an expression of a much greater global inversion. They want to delete all religions and force humanity to worship a one-world religion – Sabbatian Satanism that also includes worship of the Earth goddess. Satanic themes are being more and more introduced into mainstream society and while Christianity is currently the foremost target for destruction the others are planned to follow.

Figure 7: The Cult goddess of Babylon disguised as the Statue of Liberty holding the flame of Lucifer the 'light bringer'.

Figure 8: Liberty's mirror image in Paris where the New York version originated.

Marx brothers

Rabbi Marvin Antelman connects the Illuminati to the Jacobins in *To Eliminate the Opiate* and Jacobins were the force behind the French Revolution. He links both to the Bund der Gerechten, or League of the Just, which was the network that inflicted communism/Marxism on the world. Antelman wrote:

> The original inner circle of the Bund der Gerechten consisted of born Catholics, Protestants and Jews [Sabbatian-Frankist infiltrators], and those representatives of respective subdivisions formulated schemes for the ultimate destruction of their faiths. The heretical Catholics laid plans which they felt would take a century or more for the ultimate destruction of the church; the apostate Jews for the ultimate destruction of the Jewish religion.

Sabbatian-created communism connects into this anti-religion agenda in that communism does not allow for the free practice of religion. The Sabbatian 'Bund' became the International Communist Party and Communist League and in 1848 'Marxism' was born with the Communist Manifesto of Sabbatian assets Karl Marx and Friedrich Engels. It is absolutely no coincidence that Marxism, just a different name for fascist and other centrally-controlled tyrannies, is being imposed worldwide as a result of the 'Covid' hoax and nor that Marxist/fascist China was the place where the hoax originated. The reason for this will become very clear in the chapter 'Covid: The calculated catastrophe'. The so-called 'Woke' mentality has hijacked

traditional beliefs of the political left and replaced them with far-right make-believe 'social justice' better known as Marxism. Woke will, however, be swallowed by its own perceived 'revolution' which is really the work of billionaires and billionaire corporations feigning being 'Woke'. Marxism is being touted by Wokers as a replacement for 'capitalism' when we don't have 'capitalism'. We have cartelism in which the market is stitched up by the very Cult billionaires and corporations bankrolling Woke. Billionaires love Marxism which keeps the people in servitude while they control from the top. Terminally naïve Wokers think they are 'changing the world' when it's the Cult that is doing the changing and when they have played their vital part and become surplus to requirements they, too, will be targeted. The Illuminati-Jacobins were behind the period known as 'The Terror' in the French Revolution in 1793 and 1794 when Jacobin Maximillian de Robespierre and his Orwellian 'Committee of Public Safety' killed 17,000 'enemies of the Revolution' who had once been 'friends of the Revolution'. Karl Marx (1818-1883), whose Sabbatian creed of Marxism has cost the lives of at least 100 million people, is a hero once again to Wokers who have been systematically kept ignorant of real history by their 'education' programming. As a result they now promote a Sabbatian 'Marxist' abomination destined at some point to consume them. Rabbi Antelman, who spent decades researching the Sabbatian plot, said of the League of the Just and Karl Marx:

> Contrary to popular opinion Karl Marx did not originate the Communist Manifesto. He was paid for his services by the League of the Just, which was known in its country of origin, Germany, as the Bund der Geaechteten.

Antelman said the text attributed to Marx was the work of other people and Marx 'was only repeating what others already said'. Marx was 'a hired hack – lackey of the wealthy Illuminists'. Marx famously said that religion was the 'opium of the people' (part of the Sabbatian plan to demonise religion) and Antelman called his books, *To Eliminate the Opiate*. Marx was born Jewish, but his family converted to Christianity (Sabbatian modus operandi) and he

attacked Jews, not least in his book, *A World Without Jews*. In doing so he supported the Sabbatian plan to destroy traditional Jewishness and Judaism which we are clearly seeing today with the vindictive targeting of orthodox Jews by the Sabbatian government of Israel over 'Covid' laws. I don't follow any religion and it has done much damage to the world over centuries and acted as a perceptual straightjacket. Renegade Minds, however, are always asking *why* something is being done. It doesn't matter if they agree or disagree with what is happening – *why* is it happening is the question. The 'why?' can be answered with regard to religion in that religions create interacting communities of believers when the Cult wants to dismantle all discourse, unity and interaction (see 'Covid' lockdowns) and the ultimate goal is to delete all religions for a one-world religion of Cult Satanism worshipping their 'god' of which more later. We see the same 'why?' with gun control in America. I don't have guns and don't want them, but why is the Cult seeking to disarm the population at the same time that law enforcement agencies are armed to their molars and why has every tyrant in history sought to disarm people before launching the final takeover? They include Hitler, Stalin, Pol Pot and Mao who followed confiscation with violent seizing of power. You know it's a Cult agenda by the people who immediately race to the microphones to exploit dead people in multiple shootings. Ultra-Zionist Cult lackey Senator Chuck Schumer was straight on the case after ten people were killed in Boulder, Colorado in March, 2121. Simple rule … if Schumer wants it the Cult wants it and the same with his ultra-Zionist mate the wild-eyed Senator Adam Schiff. At the same time they were calling for the disarmament of Americans, many of whom live a long way from a police response, Schumer, Schiff and the rest of these pampered clowns were sitting on Capitol Hill behind a razor-wired security fence protected by thousands of armed troops in addition to their own armed bodyguards. Mom and pop in an isolated home? They're just potential mass shooters.

Zion Mainframe

Sabbatian-Frankists and most importantly the Rothschilds were behind the creation of 'Zionism', a political movement that demanded a Jewish homeland in Israel as promised by Sabbatai Zevi. The very symbol of Israel comes from the German meaning of the name Rothschild. Dynasty founder Mayer Amschel Rothschild changed the family name from Bauer to Rothschild, or 'Red-Shield' in German, in deference to the six-pointed 'Star of David' hexagram displayed on the family's home in Frankfurt. The symbol later appeared on the flag of Israel after the Rothschilds were centrally involved in its creation. Hexagrams are not a uniquely Jewish symbol and are widely used in occult ('hidden') networks often as a symbol for Saturn (see my other books for why). Neither are Zionism and Jewishness interchangeable. Zionism is a political movement and philosophy and not a 'race' or a people. Many Jews oppose Zionism and many non-Jews, including US President Joe Biden, call themselves Zionists as does Israel-centric Donald Trump. America's support for the Israel government is pretty much a gimme with ultra-Zionist billionaires and corporations providing fantastic and dominant funding for both political parties. Former Congresswoman Cynthia McKinney has told how she was approached immediately she ran for office to 'sign the pledge' to Israel and confirm that she would always vote in that country's best interests. All American politicians are approached in this way. Anyone who refuses will get no support or funding from the enormous and all-powerful Zionist lobby that includes organisations like mega-lobby group AIPAC, the American Israel Public Affairs Committee. Trump's biggest funder was ultra-Zionist casino and media billionaire Sheldon Adelson while major funders of the Democratic Party include ultra-Zionist George Soros and ultra-Zionist financial and media mogul, Haim Saban. Some may reel back at the suggestion that Soros is an Israel-firster (Sabbatian-controlled Israel-firster), but Renegade Minds watch the actions not the words and everywhere Soros donates his billions the Sabbatian agenda benefits. In the spirit of Sabbatian inversion Soros pledged $1 billion for a new university network to promote 'liberal values and tackle intolerance'. He made the announcement during his annual speech

at the Cult-owned World Economic Forum in Davos, Switzerland, in January, 2020, after his 'harsh criticism' of 'authoritarian rulers' around the world. You can only laugh at such brazen mendacity. How *he* doesn't laugh is the mystery. Translated from the Orwellian 'liberal values and tackle intolerance' means teaching non-white people to hate white people and for white people to loathe themselves for being born white. The reason for that will become clear.

The 'Anti-Semitism' fraud

Zionists support the Jewish homeland in the land of Palestine which has been the Sabbatian-Rothschild goal for so long, but not for the benefit of Jews. Sabbatians and their global Anti-Semitism Industry have skewed public and political opinion to equate opposing the violent extremes of Zionism to be a blanket attack and condemnation of all Jewish people. Sabbatians and their global Anti-Semitism Industry have skewed public and political opinion to equate opposing the violent extremes of Zionism to be a blanket attack and condemnation of all Jewish people. This is nothing more than a Sabbatian protection racket to stop legitimate investigation and exposure of their agendas and activities. The official definition of 'anti-Semitism' has more recently been expanded to include criticism of Zionism – a *political movement* – and this was done to further stop exposure of Sabbatian infiltrators who created Zionism as we know it today in the 19th century. Renegade Minds will talk about these subjects when they know the shit that will come their way. People must decide if they want to know the truth or just cower in the corner in fear of what others will say. Sabbatians have been trying to label me as 'anti-Semitic' since the 1990s as I have uncovered more and more about their background and agendas. Useless, gutless, fraudulent 'journalists' then just repeat the smears without question and on the day I was writing this section a pair of unquestioning repeaters called Ben Quinn and Archie Bland (how appropriate) outright called me an 'anti-Semite' in the establishment propaganda sheet, the London *Guardian*, with no supporting evidence. The

Sabbatian Anti-Semitism Industry said so and who are they to question that? They wouldn't dare. Ironically 'Semitic' refers to a group of languages in the Middle East that are almost entirely Arabic. 'Anti-Semitism' becomes 'anti-Arab' which if the consequences of this misunderstanding were not so grave would be hilarious. Don't bother telling Quinn and Bland. I don't want to confuse them, bless 'em. One reason I am dubbed 'anti-Semitic' is that I wrote in the 1990s that Jewish operatives (Sabbatians) were heavily involved in the Russian Revolution when Sabbatians overthrew the Romanov dynasty. This apparently made me 'anti-Semitic'. Oh, really? Here is a section from *The Trigger*:

British journalist Robert Wilton confirmed these themes in his 1920 book *The Last Days of the Romanovs* when he studied official documents from the Russian government to identify the members of the Bolshevik ruling elite between 1917 and 1919. The Central Committee included 41 Jews among 62 members; the Council of the People's Commissars had 17 Jews out of 22 members; and 458 of the 556 most important Bolshevik positions between 1918 and 1919 were occupied by Jewish people. Only 17 were Russian. Then there were the 23 Jews among the 36 members of the vicious Cheka Soviet secret police established in 1917 who would soon appear all across the country.

Professor Robert Service of Oxford University, an expert on 20th century Russian history, found evidence that ['Jewish'] Leon Trotsky had sought to make sure that Jews were enrolled in the Red Army and were disproportionately represented in the Soviet civil bureaucracy that included the Cheka which performed mass arrests, imprisonment and executions of 'enemies of the people'. A US State Department Decimal File (861.00/5339) dated November 13th, 1918, names [Rothschild banking agent in America] Jacob Schiff and a list of ultra-Zionists as funders of the Russian Revolution leading to claims of a 'Jewish plot', but the key point missed by all is they were not 'Jews' – they were Sabbatian-Frankists.

Britain's Winston Churchill made the same error by mistake or otherwise. He wrote in a 1920 edition of the *Illustrated Sunday Herald* that those behind the Russian revolution were part of a 'worldwide conspiracy for the overthrow of civilisation and for the reconstitution of society on the basis of arrested development, of envious malevolence, and impossible equality' (see 'Woke' today because that has been created by the same network). Churchill said there was no need to exaggerate the part played in the creation of Bolshevism and in the actual bringing about of the Russian

Revolution 'by these international and for the most part atheistical Jews' ['atheistical Jews' = Sabbatians]. Churchill said it is certainly a very great one and probably outweighs all others: 'With the notable exception of Lenin, the majority of the leading figures are Jews.' He went on to describe, knowingly or not, the Sabbatian modus operandi of placing puppet leaders nominally in power while they control from the background:

> Moreover, the principal inspiration and driving power comes from the Jewish leaders. Thus Tchitcherin, a pure Russian, is eclipsed by his nominal subordinate, Litvinoff, and the influence of Russians like Bukharin or Lunacharski cannot be compared with the power of Trotsky, or of Zinovieff, the Dictator of the Red Citadel (Petrograd), or of Krassin or Radek – all Jews. In the Soviet institutions the predominance of Jews is even more astonishing. And the prominent, if not indeed the principal, part in the system of terrorism applied by the Extraordinary Commissions for Combatting Counter-Revolution has been taken by Jews, and in some notable cases by Jewesses.

What I said about seriously disproportionate involvement in the Russian Revolution by Jewish 'revolutionaries' (Sabbatians) is provable fact, but truth is no defence against the Sabbatian Anti-Semitism Industry, its repeater parrots like Quinn and Bland, and the now breathtaking network of so-called 'Woke' 'anti-hate' groups with interlocking leaderships and funding which have the role of discrediting and silencing anyone who gets too close to exposing the Sabbatians. We have seen 'truth is no defence' confirmed in legal judgements with the Saskatchewan Human Rights Commission in Canada decreeing this: 'Truthful statements can be presented in a manner that would meet the definition of hate speech, and not all truthful statements must be free from restriction.' Most 'anti-hate' activists, who are themselves consumed by hatred, are too stupid and ignorant of the world to know how they are being used. They are far too far up their own virtue-signalling arses and it's far too dark for them to see anything.

The 'revolution' game

The background and methods of the 'Russian' Revolution are straight from the Sabbatian playbook seen in the French Revolution

and endless others around the world that appear to start as a revolution of the people against tyrannical rule and end up with a regime change to more tyrannical rule overtly or covertly. Wars, terror attacks and regime overthrows follow the Sabbatian cult through history with its agents creating them as Problem-Reaction-Solutions to remove opposition on the road to world domination. Sabbatian dots connect the Rothschilds with the Illuminati, Jacobins of the French Revolution, the 'Bund' or League of the Just, the International Communist Party, Communist League and the Communist Manifesto of Karl Marx and Friedrich Engels that would lead to the Rothschild-funded Russian Revolution. The sequence comes under the heading of 'creative destruction' when you advance to your global goal by continually destroying the status quo to install a new status quo which you then also destroy. The two world wars come to mind. With each new status quo you move closer to your planned outcome. Wars and mass murder are to Sabbatians a collective blood sacrifice ritual. They are obsessed with death for many reasons and one is that death is an inversion of life. Satanists and Sabbatians are obsessed with death and often target churches and churchyards for their rituals. Inversion-obsessed Sabbatians explain the use of inverted symbolism including the *inverted* pentagram and *inverted* cross. The inversion of the cross has been related to targeting Christianity, but the cross was a religious symbol long before Christianity and its inversion is a statement about the Sabbatian mentality and goals more than any single religion.

Sabbatians operating in Germany were behind the rise of the occult-obsessed Nazis and the subsequent Jewish exodus from Germany and Europe to Palestine and the United States after World War Two. The Rothschild dynasty was at the forefront of this both as political manipulators and by funding the operation. Why would Sabbatians help to orchestrate the horrors inflicted on Jews by the Nazis and by Stalin after they organised the Russian Revolution? Sabbatians hate Jews and their religion, that's why. They pose as Jews and secure positions of control within Jewish society and play the 'anti-Semitism' card to protect themselves from exposure

through a global network of organisations answering to the Sabbatian-created-and-controlled globe-spanning intelligence network that involves a stunning web of military-intelligence operatives and operations for a tiny country of just nine million. Among them are Jewish assets who are not Sabbatians but have been convinced by them that what they are doing is for the good of Israel and the Jewish community to protect them from what they have been programmed since childhood to believe is a Jew-hating hostile world. The Jewish community is just a highly convenient cover to hide the true nature of Sabbatians. Anyone getting close to exposing their game is accused by Sabbatian place-people and gofers of 'anti-Semitism' and claiming that all Jews are part of a plot to take over the world. I am not saying that. I am saying that Sabbatians – the *real* Jew-haters – have infiltrated the Jewish community to use them both as a cover and an 'anti-Semitic' defence against exposure. Thus we have the Anti-Semitism Industry targeted researchers in this way and most Jewish people think this is justified and genuine. They don't know that their 'Jewish' leaders and institutions of state, intelligence and military are not controlled by Jews at all, but cultists and stooges of Sabbatian-Frankism. I once added my name to a pro-Jewish freedom petition online and the next time I looked my name was gone and text had been added to the petition blurb to attack me as an 'anti-Semite' such is the scale of perceptual programming.

Moving on America

I tell the story in *The Trigger* and a chapter called 'Atlantic Crossing' how particularly after Israel was established the Sabbatians moved in on the United States and eventually grasped control of government administration, the political system via both Democrats and Republicans, the intelligence community like the CIA and National Security Agency (NSA), the Pentagon and mass media. Through this seriously compartmentalised network Sabbatians and their operatives in Mossad, Israeli Defense Forces (IDF) and US agencies pulled off 9/11 and blamed it on 19 'Al-Qaeda hijackers' dominated by men from, or connected to, Sabbatian-ruled Saudi

Arabia. The '19' were not even on the planes let alone flew those big passenger jets into buildings while being largely incompetent at piloting one-engine light aircraft. 'Hijacker' Hani Hanjour who is said to have flown American Airlines Flight 77 into the Pentagon with a turn and manoeuvre most professional pilots said they would have struggled to do was banned from renting a small plane by instructors at the Freeway Airport in Bowie, Maryland, just *six weeks* earlier on the grounds that he was an incompetent pilot. The Jewish population of the world is just 0.2 percent with even that almost entirely concentrated in Israel (75 percent Jewish) and the United States (around two percent). This two percent and globally 0.2 percent refers to *Jewish* people and not Sabbatian interlopers who are a fraction of that fraction. What a sobering thought when you think of the fantastic influence on world affairs of tiny Israel and that the Project for the New America Century (PNAC) which laid out the blueprint in September, 2000, for America's war on terror and regime change wars in Iraq, Libya and Syria was founded and dominated by Sabbatians known as 'Neocons'. The document conceded that this plan would not be supported politically or publicly without a major attack on American soil and a Problem-Reaction-Solution excuse to send troops to war across the Middle East. Sabbatian Neocons said:

> ... [The] process of transformation ... [war and regime change] ... is likely to be a long one, absent some catastrophic and catalysing event – like a new Pearl Harbor.

Four months later many of those who produced that document came to power with their inane puppet George Bush from the long-time Sabbatian Bush family. They included Sabbatian Dick Cheney who was officially vice-president, but really de-facto president for the entirety of the 'Bush' government. Nine months after the 'Bush' inauguration came what Bush called at the time 'the Pearl Harbor of the 21st century' and with typical Sabbatian timing and symbolism 2001 was the 60th anniversary of the attack in 1941 by the Japanese Air Force on Pearl Harbor, Hawaii, which allowed President Franklin Delano Roosevelt to take the United States into a Sabbatian-

instigated Second World War that he said in his election campaign that he never would. The evidence is overwhelming that Roosevelt and his military and intelligence networks knew the attack was coming and did nothing to stop it, but they did make sure that America's most essential naval ships were not in Hawaii at the time. Three thousand Americans died in the Pearl Harbor attacks as they did on September 11th. By the 9/11 year of 2001 Sabbatians had widely infiltrated the US government, military and intelligence operations and used their compartmentalised assets to pull off the 'Al-Qaeda' attacks. If you read *The Trigger* it will blow your mind to see the utterly staggering concentration of 'Jewish' operatives (Sabbatian infiltrators) in essential positions of political, security, legal, law enforcement, financial and business power before, during, and after the attacks to make them happen, carry them out, and then cover their tracks – and I do mean *staggering* when you think of that 0.2 percent of the world population and two percent of Americans which are Jewish while Sabbatian infiltrators are a fraction of that. A central foundation of the 9/11 conspiracy was the hijacking of government, military, Air Force and intelligence computer systems in real time through 'back-door' access made possible by Israeli (Sabbatian) 'cyber security' software. Sabbatian-controlled Israel is on the way to rivalling Silicon Valley for domination of cyberspace and is becoming the dominant force in cyber-security which gives them access to entire computer systems and their passcodes across the world. Then add to this that Zionists head (officially) Silicon Valley giants like Google (Larry Page and Sergey Brin), Google-owned YouTube (Susan Wojcicki), Facebook (Mark Zuckerberg and Sheryl Sandberg), and Apple (Chairman Arthur D. Levinson), and that ultra-Zionist hedge fund billionaire Paul Singer has a $1 billion stake in Twitter which is only nominally headed by 'CEO' pothead Jack Dorsey. As cable news host Tucker Carlson said of Dorsey: 'There used to be debate in the medical community whether dropping a ton of acid had permanent effects and I think that debate has now ended.' Carlson made the comment after Dorsey told a hearing on Capitol Hill (if you cut through his bullshit) that he

believed in free speech so long as he got to decide what you can hear and see. These 'big names' of Silicon Valley are only front men and women for the Global Cult, not least the Sabbatians, who are the true controllers of these corporations. Does anyone still wonder why these same people and companies have been ferociously censoring and banning people (like me) for exposing any aspect of the Cult agenda and especially the truth about the 'Covid' hoax which Sabbatians have orchestrated?

The Jeffrey Epstein paedophile ring was a Sabbatian operation. He was officially 'Jewish' but he was a Sabbatian and women abused by the ring have told me about the high number of 'Jewish' people involved. The Epstein horror has Sabbatian written all over it and matches perfectly their modus operandi and obsession with sex and ritual. Epstein was running a Sabbatian blackmail ring in which famous people with political and other influence were provided with young girls for sex while everything was being filmed and recorded on hidden cameras and microphones at his New York house, Caribbean island and other properties. Epstein survivors have described this surveillance system to me and some have gone public. Once the famous politician or other figure knew he or she was on video they tended to do whatever they were told. Here we go again …when you've got them by the balls their hearts and minds will follow. Sabbatians use this blackmail technique on a wide scale across the world to entrap politicians and others they need to act as demanded. Epstein's private plane, the infamous 'Lolita Express', had many well-known passengers including Bill Clinton while Bill Gates has flown on an Epstein plane and met with him four years after Epstein had been jailed for paedophilia. They subsequently met many times at Epstein's home in New York according to a witness who was there. Epstein's infamous side-kick was Ghislaine Maxwell, daughter of Mossad agent and ultra-Zionist mega-crooked British businessman, Bob Maxwell, who at one time owned the *Daily Mirror* newspaper. Maxwell was murdered at sea on his boat in 1991 by Sabbatian-controlled Mossad when he became a liability with his

business empire collapsing as a former Mossad operative has confirmed (see *The Trigger*).

Money, money, money, funny money …

Before I come to the Sabbatian connection with the last three US presidents I will lay out the crucial importance to Sabbatians of controlling banking and finance. Sabbatian Mayer Amschel Rothschild set out to dominate this arena in his family's quest for total global control. What is freedom? It is, in effect, choice. The more choices you have the freer you are and the fewer your choices the more you are enslaved. In the global structure created over centuries by Sabbatians the biggest decider and restrictor of choice is … money. Across the world if you ask people what they would like to do with their lives and why they are not doing that they will reply 'I don't have the money'. This is the idea. A global elite of multi-billionaires are described as 'greedy' and that is true on one level; but control of money – who has it and who doesn't – is not primarily about greed. It's about control. Sabbatians have seized ever more control of finance and sucked the wealth of the world out of the hands of the population. We talk now, after all, about the 'One-percent' and even then the wealthiest are a lot fewer even than that. This has been made possible by a money scam so outrageous and so vast it could rightly be called the scam of scams founded on creating 'money' out of nothing and 'loaning' that with interest to the population. Money out of nothing is called 'credit'. Sabbatians have asserted control over governments and banking ever more completely through the centuries and secured financial laws that allow banks to lend hugely more than they have on deposit in a confidence trick known as fractional reserve lending. Imagine if you could lend money that doesn't exist and charge the recipient interest for doing so. You would end up in jail. Bankers by contrast end up in mansions, private jets, Malibu and Monaco.

Banks are only required to keep a fraction of their deposits and wealth in their vaults and they are allowed to lend 'money' they don't have called 'credit. Go into a bank for a loan and if you succeed

the banker will not move any real wealth into your account. They will type into your account the amount of the agreed 'loan' – say £100,000. This is not wealth that really exists; it is non-existent, fresh-air, created-out-of-nothing 'credit' which has never, does not, and will never exist except in theory. Credit is backed by nothing except wind and only has buying power because people think that it has buying power and accept it in return for property, goods and services. I have described this situation as like those cartoon characters you see chasing each other and when they run over the edge of a cliff they keep running forward on fresh air until one of them looks down, realises what's happened, and they all crash into the ravine. The whole foundation of the Sabbatian financial system is to stop people looking down except for periodic moments when they want to crash the system (as in 2008 and 2020 ongoing) and reap the rewards from all the property, businesses and wealth their borrowers had signed over as 'collateral' in return for a 'loan' of fresh air. Most people think that money is somehow created by governments when it comes into existence from the start as a debt through banks 'lending' illusory money called credit. Yes, the very currency of exchange is a *debt* from day one issued as an interest-bearing loan. Why don't governments create money interest-free and lend it to their people interest-free? Governments are controlled by Sabbatians and the financial system is controlled by Sabbatians for whom interest-free money would be a nightmare come true. Sabbatians underpin their financial domination through their global network of central banks, including the privately-owned US Federal Reserve and Britain's Bank of England, and this is orchestrated by a privately-owned central bank coordination body called the Bank for International Settlements in Basle, Switzerland, created by the usual suspects including the Rockefellers and Rothschilds. Central bank chiefs don't answer to governments or the people. They answer to the Bank for International Settlements or, in other words, the Global Cult which is dominated today by Sabbatians.

Built-in disaster

There are so many constituent scams within the overall banking scam. When you take out a loan of thin-air credit only the amount of that loan is theoretically brought into circulation to add to the amount in circulation; but you are paying back the principle plus interest. The additional interest is not created and this means that with every 'loan' there is a shortfall in the money in circulation between what is borrowed and what has to be paid back. There is never even close to enough money in circulation to repay all outstanding public and private debt including interest. Coldly weaved in the very fabric of the system is the certainty that some will lose their homes, businesses and possessions to the banking 'lender'. This is less obvious in times of 'boom' when the amount of money in circulation (and the debt) is expanding through more people wanting and getting loans. When a downturn comes and the money supply contracts it becomes painfully obvious that there is not enough money to service all debt and interest. This is less obvious in times of 'boom' when the amount of money in circulation (and the debt) is expanding through more people wanting and getting loans. When a downturn comes and the money supply contracts and it becomes painfully obvious – as in 2008 and currently – that there is not enough money to service all debt and interest. Sabbatian banksters have been leading the human population through a calculated series of booms (more debt incurred) and busts (when the debt can't be repaid and the banks get the debtor's tangible wealth in exchange for non-existent 'credit'). With each 'bust' Sabbatian bankers have absorbed more of the world's tangible wealth and we end up with the One-percent. Governments are in bankruptcy levels of debt to the same system and are therefore owned by a system they do not control. The Federal Reserve, 'America's central bank', is privately-owned and American presidents only nominally appoint its chairman or woman to maintain the illusion that it's an arm of government. It's not. The 'Fed' is a cartel of private banks which handed billions to its associates and friends after the crash of 2008 and has been Sabbatian-controlled since it was manipulated into being in 1913 through the covert trickery of Rothschild banking agents Jacob Schiff and Paul

Warburg, and the Sabbatian Rockefeller family. Somehow from a Jewish population of two-percent and globally 0.2 percent (Sabbatian interlopers remember are far smaller) ultra-Zionists headed the Federal Reserve for 31 years between 1987 and 2018 in the form of Alan Greenspan, Bernard Bernanke and Janet Yellen (now Biden's Treasury Secretary) with Yellen's deputy chairman a Israeli-American duel citizen and ultra-Zionist Stanley Fischer, a former governor of the Bank of Israel. Ultra-Zionist Fed chiefs spanned the presidencies of Ronald Reagan ('Republican'), Father George Bush ('Republican'), Bill Clinton ('Democrat'), Boy George Bush ('Republican') and Barack Obama ('Democrat'). We should really add the pre-Greenspan chairman, Paul Adolph Volcker, 'appointed' by Jimmy Carter ('Democrat') who ran the Fed between 1979 and 1987 during the Carter and Reagan administrations before Greenspan took over. Volcker was a long-time associate and business partner of the Rothschilds. No matter what the 'party' officially in power the United States economy was directed by the same force. Here are members of the Obama, Trump and Biden administrations and see if you can make out a common theme.

Barack Obama ('Democrat')

Ultra-Zionists Robert Rubin, Larry Summers, and Timothy Geithner ran the US Treasury in the Clinton administration and two of them reappeared with Obama. Ultra-Zionist Fed chairman Alan Greenspan had manipulated the crash of 2008 through deregulation and jumped ship just before the disaster to make way for ultra-Zionist Bernard Bernanke to hand out trillions to Sabbatian 'too big to fail' banks and businesses, including the ubiquitous ultra-Zionist Goldman Sachs which has an ongoing staff revolving door operation between itself and major financial positions in government worldwide. Obama inherited the fallout of the crash when he took office in January, 2009, and fortunately he had the support of his ultra-Zionist White House Chief of Staff Rahm Emmanuel, son of a terrorist who helped to bomb Israel into being in 1948, and his ultra-Zionist senior adviser David Axelrod, chief strategist in Obama's two

successful presidential campaigns. Emmanuel, later mayor of Chicago and former senior fundraiser and strategist for Bill Clinton, is an example of the Sabbatian policy after Israel was established of migrating insider families to America so their children would be born American citizens. 'Obama' chose this financial team throughout his administration to respond to the Sabbatian-instigated crisis:

Timothy Geithner (ultra-Zionist) Treasury Secretary; Jacob J. Lew, Treasury Secretary; Larry Summers (ultra-Zionist), director of the White House National Economic Council; Paul Adolph Volcker (Rothschild business partner), chairman of the Economic Recovery Advisory Board; Peter Orszag (ultra-Zionist), director of the Office of Management and Budget overseeing all government spending; Penny Pritzker (ultra-Zionist), Commerce Secretary; Jared Bernstein (ultra-Zionist), chief economist and economic policy adviser to Vice President Joe Biden; Mary Schapiro (ultra-Zionist), chair of the Securities and Exchange Commission (SEC); Gary Gensler (ultra-Zionist), chairman of the Commodity Futures Trading Commission (CFTC); Sheila Bair (ultra-Zionist), chair of the Federal Deposit Insurance Corporation (FDIC); Karen Mills (ultra-Zionist), head of the Small Business Administration (SBA); Kenneth Feinberg (ultra-Zionist), Special Master for Executive [bail-out] Compensation. Feinberg would be appointed to oversee compensation (with strings) to 9/11 victims and families in a campaign to stop them having their day in court to question the official story. At the same time ultra-Zionist Bernard Bernanke was chairman of the Federal Reserve and these are only some of the ultra-Zionists with allegiance to Sabbatian-controlled Israel in the Obama government. Obama's biggest corporate donor was ultra-Zionist Goldman Sachs which had employed many in his administration.

Donald Trump ('Republican')

Trump claimed to be an outsider (he wasn't) who had come to 'drain the swamp'. He embarked on this goal by immediately appointing ultra-Zionist Steve Mnuchin, a Goldman Sachs employee for 17

years, as his Treasury Secretary. Others included Gary Cohn (ultra-Zionist), chief operating officer of Goldman Sachs, his first Director of the National Economic Council and chief economic adviser, who was later replaced by Larry Kudlow (ultra-Zionist). Trump's senior adviser throughout his four years in the White House was his sinister son-in-law Jared Kushner, a life-long friend of Israel Prime Minister Benjamin Netanyahu. Kushner is the son of a convicted crook who was pardoned by Trump in his last days in office. Other ultra-Zionists in the Trump administration included: Stephen Miller, Senior Policy Adviser; Avrahm Berkowitz, Deputy Adviser to Trump and his Senior Adviser Jared Kushner; Ivanka Trump, Adviser to the President, who converted to Judaism when she married Jared Kushner; David Friedman, Trump lawyer and Ambassador to Israel; Jason Greenblatt, Trump Organization executive vice president and chief legal officer, who was made Special Representative for International Negotiations and the Israeli-Palestinian Conflict; Rod Rosenstein, Deputy Attorney General; Elliot Abrams, Special Representative for Venezuela, then Iran; John Eisenberg, National Security Council Legal Adviser and Deputy Council to the President for National Security Affairs; Anne Neuberger, Deputy National Manager, National Security Agency; Ezra Cohen-Watnick, Acting Under Secretary of Defense for Intelligence; Elan Carr, Special Envoy to monitor and combat anti-Semitism; Len Khodorkovsky, Deputy Special Envoy to monitor and combat anti-Semitism; Reed Cordish, Assistant to the President, Intragovernmental and Technology Initiatives. Trump Vice President Mike Pence and Secretary of State Mike Pompeo, both Christian Zionists, were also vehement supporters of Israel and its goals and ambitions.

Donald 'free-speech believer' Trump pardoned a number of financial and violent criminals while ignoring calls to pardon Julian Assange and Edward Snowden whose crimes are revealing highly relevant information about government manipulation and corruption and the widespread illegal surveillance of the American people by US 'security' agencies. It's so good to know that Trump is on the side of freedom and justice and not mega-criminals with

allegiance to Sabbatian-controlled Israel. These included a pardon for Israeli spy Jonathan Pollard who was jailed for life in 1987 under the Espionage Act. Aviem Sella, the Mossad agent who recruited Pollard, was also pardoned by Trump while Assange sat in jail and Snowden remained in exile in Russia. Sella had 'fled' (was helped to escape) to Israel in 1987 and was never extradited despite being charged under the Espionage Act. A Trump White House statement said that Sella's clemency had been 'supported by Benjamin Netanyahu, Ron Dermer, Israel's US Ambassador, David Friedman, US Ambassador to Israel and Miriam Adelson, wife of leading Trump donor Sheldon Adelson who died shortly before. Other friends of Jared Kushner were pardoned along with Sholom Weiss who was believed to be serving the longest-ever white-collar prison sentence of more than 800 years in 2000. The sentence was commuted of Ponzi-schemer Eliyahu Weinstein who defrauded Jews and others out of $200 million. I did mention that Assange and Snowden were ignored, right? Trump gave Sabbatians almost everything they asked for in military and political support, moving the US Embassy from Tel Aviv to Jerusalem with its critical symbolic and literal implications for Palestinian statehood, and the 'deal of the Century' designed by Jared Kushner and David Friedman which gave the Sabbatian Israeli government the green light to substantially expand its already widespread program of building illegal Jewish-only settlements in the occupied land of the West Bank. This made a two-state 'solution' impossible by seizing all the land of a potential Palestinian homeland and that had been the plan since 1948 and then 1967 when the Arab-controlled Gaza Strip, West Bank, Sinai Peninsula and Syrian Golan Heights were occupied by Israel. All the talks about talks and road maps and delays have been buying time until the West Bank was physically occupied by Israeli real estate. Trump would have to be a monumentally ill-informed idiot not to see that this was the plan he was helping to complete. The Trump administration was in so many ways the Kushner administration which means the Netanyahu administration which means the Sabbatian administration. I understand why many opposing Cult fascism in all its forms gravitated to Trump, but he

was a crucial part of the Sabbatian plan and I will deal with this in the next chapter.

Joe Biden ('Democrat')

A barely cognitive Joe Biden took over the presidency in January, 2021, along with his fellow empty shell, Vice-President Kamala Harris, as the latest Sabbatian gofers to enter the White House. Names on the door may have changed and the 'party' – the force behind them remained the same as Zionists were appointed to a stream of pivotal areas relating to Sabbatian plans and policy. They included: Janet Yellen, Treasury Secretary, former head of the Federal Reserve, and still another ultra-Zionist running the US Treasury after Mnuchin (Trump), Lew and Geithner (Obama), and Summers and Rubin (Clinton); Anthony Blinken, Secretary of State; Wendy Sherman, Deputy Secretary of State (so that's 'Biden's' Sabbatian foreign policy sorted); Jeff Zients, White House coronavirus coordinator; Rochelle Walensky, head of the Centers for Disease Control; Rachel Levine, transgender deputy health secretary (that's 'Covid' hoax policy under control); Merrick Garland, Attorney General; Alejandro Mayorkas, Secretary of Homeland Security; Cass Sunstein, Homeland Security with responsibility for new immigration laws; Avril Haines, Director of National Intelligence; Anne Neuberger, National Security Agency cybersecurity director (note, cybersecurity); David Cohen, CIA Deputy Director; Ronald Klain, Biden's Chief of Staff (see Rahm Emanuel); Eric Lander, a 'leading geneticist', Office of Science and Technology Policy director (see Smart Grid, synthetic biology agenda); Jessica Rosenworcel, acting head of the Federal Communications Commission (FCC) which controls Smart Grid technology policy and electromagnetic communication systems including 5G. How can it be that so many pivotal positions are held by two-percent of the American population and 0.2 percent of the world population administration after administration no matter who is the president and what is the party? It's a coincidence? Of course it's not and this is why Sabbatians have built their colossal global web of interlocking 'anti-

hate' hate groups to condemn anyone who asks these glaring questions as an 'anti-Semite'. The way that Jewish people horrifically abused in Sabbatian-backed Nazi Germany are exploited to this end is stomach-turning and disgusting beyond words.

Political fusion

Sabbatian manipulation has reversed the roles of Republicans and Democrats and the same has happened in Britain with the Conservative and Labour Parties. Republicans and Conservatives were always labelled the 'right' and Democrats and Labour the 'left', but look at the policy positions now and the Democrat-Labour 'left' has moved further to the 'right' than Republicans and Conservatives under the banner of 'Woke', the Cult-created far-right tyranny. Where once the Democrat-Labour 'left' defended free speech and human rights they now seek to delete them and as I said earlier despite the 'Covid' fascism of the Jackboot Johnson Conservative government in the UK the Labour Party of leader Keir Starmer demanded even more extreme measures. The Labour Party has been very publicly absorbed by Sabbatians after a political and media onslaught against the previous leader, the weak and inept Jeremy Corbyn, over made-up allegations of 'anti-Semitism' both by him and his party. The plan was clear with this 'anti-Semite' propaganda and what was required in response was a swift and decisive 'fuck off' from Corbyn and a statement to expose the Anti-Semitism Industry (Sabbatian) attempt to silence Labour criticism of the Israeli government (Sabbatians) and purge the party of all dissent against the extremes of ultra-Zionism (Sabbatians). Instead Corbyn and his party fell to their knees and appeased the abusers which, by definition, is impossible. Appeasing one demand leads only to a new demand to be appeased until takeover is complete. Like I say – 'fuck off' would have been a much more effective policy and I have used it myself with great effect over the years when Sabbatians are on my case which is most of the time. I consider that fact a great compliment, by the way. The outcome of the Labour Party capitulation is that we now have a Sabbatian-controlled

Conservative Party 'opposed' by a Sabbatian-controlled Labour Party in a one-party Sabbatian state that hurtles towards the extremes of tyranny (the Sabbatian cult agenda). In America the situation is the same. Labour's Keir Starmer spends his days on his knees with his tongue out pointing to Tel Aviv, or I guess now Jerusalem, while Boris Johnson has an 'anti-Semitism czar' in the form of former Labour MP John Mann who keeps Starmer company on his prayer mat.

Sabbatian influence can be seen in Jewish members of the Labour Party who have been ejected for criticism of Israel including those from families that suffered in Nazi Germany. Sabbatians despise real Jewish people and target them even more harshly because it is so much more difficult to dub them 'anti-Semitic' although in their desperation they do try.

CHAPTER THREE

The Pushbacker sting

Until you realize how easy it is for your mind to be manipulated, you remain the puppet of someone else's game
Evita Ochel

I will use the presidencies of Trump and Biden to show how the manipulation of the one-party state plays out behind the illusion of political choice across the world. No two presidencies could – on the face of it – be more different and apparently at odds in terms of direction and policy.

A Renegade Mind sees beyond the obvious and focuses on outcomes and consequences and not image, words and waffle. The Cult embarked on a campaign to divide America between those who blindly support its agenda (the mentality known as 'Woke') and those who are pushing back on where the Cult and its Sabbatians want to go. This presents infinite possibilities for dividing and ruling the population by setting them at war with each other and allows a perceptual ring fence of demonisation to encircle the Pushbackers in a modern version of the Little Big Horn in 1876 when American cavalry led by Lieutenant Colonel George Custer were drawn into a trap, surrounded and killed by Native American tribes defending their land of thousands of years from being seized by the government. In this modern version the roles are reversed and it's those defending themselves from the Sabbatian government who are surrounded and the government that's seeking to destroy them. This trap was set years ago and to explain how we must return to 2016

and the emergence of Donald Trump as a candidate to be President of the United States. He set out to overcome the best part of 20 other candidates in the Republican Party before and during the primaries and was not considered by many in those early stages to have a prayer of living in the White House. The Republican Party was said to have great reservations about Trump and yet somehow he won the nomination. When you know how American politics works – politics in general – there is no way that Trump could have become the party's candidate unless the Sabbatian-controlled 'Neocons' that run the Republican Party wanted that to happen. We saw the proof in emails and documents made public by WikiLeaks that the Democratic Party hierarchy, or Democons, systematically undermined the campaign of Bernie Sanders to make sure that Sabbatian gofer Hillary Clinton won the nomination to be their presidential candidate. If the Democons could do that then the Neocons in the Republican Party could have derailed Trump in the same way. But they didn't and at that stage I began to conclude that Trump could well be the one chosen to be president. If that was the case the 'why' was pretty clear to see – the goal of dividing America between Cult agenda-supporting Wokers and Pushbackers who gravitated to Trump because he was telling them what they wanted to hear. His constituency of support had been increasingly ignored and voiceless for decades and profoundly through the eight years of Sabbatian puppet Barack Obama. Now here was someone speaking their language of pulling back from the incessant globalisation of political and economic power, the exporting of American jobs to China and elsewhere by 'American' (Sabbatian) corporations, the deletion of free speech, and the mass immigration policies that had further devastated job opportunities for the urban working class of all races and the once American heartlands of the Midwest.

Beware the forked tongue

Those people collectively sighed with relief that at last a political leader was apparently on their side, but another trait of the Renegade Mind is that you look even harder at people telling you

what you want to hear than those who are telling you otherwise. Obviously as I said earlier people wish what they want to hear to be true and genuine and they are much more likely to believe that than someone saying what they don't want to here and don't want to be true. Sales people are taught to be skilled in eliciting by calculated questioning what their customers want to hear and repeating that back to them as their own opinion to get their targets to like and trust them. Assets of the Cult are also sales people in the sense of selling perception. To read Cult manipulation you have to play the long and expanded game and not fall for the Vaudeville show of party politics. Both American parties are vehicles for the Cult and they exploit them in different ways depending on what the agenda requires at that moment. Trump and the Republicans were used to be the focus of dividing America and isolating Pushbackers to open the way for a Biden presidency to become the most extreme in American history by advancing the full-blown Woke (Cult) agenda with the aim of destroying and silencing Pushbackers now labelled Nazi Trump supporters and white supremacists.

Sabbatians wanted Trump in office for the reasons described by ultra-Zionist Saul Alinsky (1909-1972) who was promoting the Woke philosophy through 'community organising' long before anyone had heard of it. In those days it still went by its traditional name of Marxism. The reason for the manipulated Trump phenomenon was laid out in Alinsky's 1971 book, *Rules for Radicals*, which was his blueprint for overthrowing democratic and other regimes and replacing them with Sabbatian Marxism. Not surprisingly his to-do list was evident in the Sabbatian French and Russian 'Revolutions' and that in China which will become very relevant in the next chapter about the 'Covid' hoax. Among Alinsky's followers have been the deeply corrupt Barack Obama, House Speaker Nancy Pelosi and Hillary Clinton who described him as a 'hero'. All three are Sabbatian stooges with Pelosi personifying the arrogant corrupt idiocy that so widely fronts up for the Cult inner core. Predictably as a Sabbatian advocate of the 'light-bringer' Alinsky features Lucifer on the dedication page of his book as the original radical who gained

his own kingdom ('Earth' as we shall see). One of Alinsky's golden radical rules was to pick an individual and focus all attention, hatred and blame on them and not to target faceless bureaucracies and corporations. *Rules for Radicals* is really a Sabbatian handbook with its contents repeatedly employed all over the world for centuries and why wouldn't Sabbatians bring to power their designer-villain to be used as the individual on which all attention, hatred and blame was bestowed? This is what they did and the only question for me is how much Trump knew that and how much he was manipulated. A bit of both, I suspect. This was Alinsky's Trump technique from a man who died in 1972. The technique has spanned history:

Pick the target, freeze it, personalize it, polarize it. Don't try to attack abstract corporations or bureaucracies. Identify a responsible individual. Ignore attempts to shift or spread the blame.

From the moment Trump came to illusory power everything was about him. It wasn't about Republican policy or opinion, but all about Trump. Everything he did was presented in negative, derogatory and abusive terms by the Sabbatian-dominated media led by Cult operations such as CNN, MSNBC, *The New York Times* and the Jeff Bezos-owned *Washington Post* – 'Pick the target, freeze it, personalize it, polarize it.' Trump was turned into a demon to be vilified by those who hated him and a demi-god loved by those who worshipped him. This, in turn, had his supporters, too, presented as equally demonic in preparation for the punchline later down the line when Biden was about to take office. It was here's a Trump, there's a Trump, everywhere a Trump, Trump. Virtually every news story or happening was filtered through the lens of 'The Donald'. You loved him or hated him and which one you chose was said to define you as Satan's spawn or a paragon of virtue. Even supporting some Trump policies or statements and not others was enough for an assault on your character. No shades of grey were or are allowed. Everything is black and white (literally and figuratively). A Californian I knew had her head utterly scrambled by her hatred for Trump while telling people they should love each other. She was so totally consumed by

Trump Derangement Syndrome as it became to be known that this glaring contradiction would never have occurred to her. By definition anyone who criticised Trump or praised his opponents was a hero and this lady described Joe Biden as 'a kind, honest gentleman' when he's a provable liar, mega-crook and vicious piece of work to boot. Sabbatians had indeed divided America using Trump as the fall-guy and all along the clock was ticking on the consequences for his supporters.

In hock to his masters

Trump gave Sabbatians via Israel almost everything they wanted in his four years. Ask and you shall receive was the dynamic between himself and Benjamin Netanyahu orchestrated by Trump's ultra-Zionist son-in-law Jared Kushner, his ultra-Zionist Ambassador to Israel, David Friedman, and ultra-Zionist 'Israel adviser', Jason Greenblatt. The last two were central to the running and protecting from collapse of his business empire, the Trump Organisation, and colossal business failures made him forever beholding to Sabbatian networks that bailed him out. By the start of the 1990s Trump owed $4 billion to banks that he couldn't pay and almost $1billion of that was down to him personally and not his companies. This mega-disaster was the result of building two new casinos in Atlantic City and buying the enormous Taj Mahal operation which led to crippling debt payments. He had borrowed fantastic sums from 72 banks with major Sabbatian connections and although the scale of debt should have had him living in a tent alongside the highway they never foreclosed. A plan was devised to lift Trump from the mire by BT Securities Corporation and Rothschild Inc. and the case was handled by Wilber Ross who had worked for the Rothschilds for 27 years. Ross would be named US Commerce Secretary after Trump's election. Another crucial figure in saving Trump was ultra-Zionist 'investor' Carl Icahn who bought the Taj Mahal casino. Icahn was made special economic adviser on financial regulation in the Trump administration. He didn't stay long but still managed to find time to make a tidy sum of a reported $31.3 million when he sold his

holdings affected by the price of steel three days before Trump imposed a 235 percent tariff on steel imports. What amazing bits of luck these people have. Trump and Sabbatian operatives have long had a close association and his mentor and legal adviser from the early 1970s until 1986 was the dark and genetically corrupt ultra-Zionist Roy Cohn who was chief counsel to Senator Joseph McCarthy's 'communist' witch-hunt in the 1950s. *Esquire* magazine published an article about Cohn with the headline 'Don't mess with Roy Cohn'. He was described as the most feared lawyer in New York and 'a ruthless master of dirty tricks ... [with] ... more than one Mafia Don on speed dial'. Cohn's influence, contacts, support and protection made Trump a front man for Sabbatians in New York with their connections to one of Cohn's many criminal employers, the 'Russian' Sabbatian Mafia. Israel-centric media mogul Rupert Murdoch was introduced to Trump by Cohn and they started a long friendship. Cohn died in 1986 weeks after being disbarred for unethical conduct by the Appellate Division of the New York State Supreme Court. The wheels of justice do indeed run slow given the length of Cohn's crooked career.

QAnon-sense

We are asked to believe that Donald Trump with his fundamental connections to Sabbatian networks and operatives has been leading the fight to stop the Sabbatian agenda for the fascistic control of America and the world. Sure he has. A man entrapped during his years in the White House by Sabbatian operatives and whose biggest financial donor was casino billionaire Sheldon Adelson who was Sabbatian to his DNA?? Oh, do come on. Trump has been used to divide America and isolate Pushbackers on the Cult agenda under the heading of 'Trump supporters', 'insurrectionists' and 'white supremacists'. The US Intelligence/Mossad Psyop or psychological operation known as QAnon emerged during the Trump years as a central pillar in the Sabbatian campaign to lead Pushbackers into the trap set by those that wished to destroy them. I knew from the start that QAnon was a scam because I had seen the same scenario many

times before over 30 years under different names and I had written about one in particular in the books. 'Not again' was my reaction when QAnon came to the fore. The same script is pulled out every few years and a new name added to the letterhead. The story always takes the same form: 'Insiders' or 'the good guys' in the government-intelligence-military 'Deep State' apparatus were going to instigate mass arrests of the 'bad guys' which would include the Rockefellers, Rothschilds, Barack Obama, Hillary Clinton, George Soros, etc., etc. Dates are given for when the 'good guys' are going to move in, but the dates pass without incident and new dates are given which pass without incident. The central message to Pushbackers in each case is that they don't have to do anything because there is 'a plan' and it is all going to be sorted by the 'good guys' on the inside. 'Trust the plan' was a QAnon mantra when the only plan was to misdirect Pushbackers into putting their trust in a Psyop they believed to be real. Beware, beware, those who tell you what you want to hear and always check it out. Right up to Biden's inauguration QAnon was still claiming that 'the Storm' was coming and Trump would stay on as president when Biden and his cronies were arrested and jailed. It was never going to happen and of course it didn't, but what did happen as a result provided that punchline to the Sabbatian Trump/QAnon Psyop.

On January 6th, 2021, a very big crowd of Trump supporters gathered in the National Mall in Washington DC down from the Capitol Building to protest at what they believed to be widespread corruption and vote fraud that stopped Trump being re-elected for a second term as president in November, 2020. I say as someone that does not support Trump or Biden that the evidence is clear that major vote-fixing went on to favour Biden, a man with cognitive problems so advanced he can often hardly string a sentence together without reading the words written for him on the Teleprompter. Glaring ballot discrepancies included serious questions about electronic voting machines that make vote rigging a comparative cinch and hundreds of thousands of paper votes that suddenly appeared during already advanced vote counts and virtually all of

them for Biden. Early Trump leads in crucial swing states suddenly began to close and disappear. The pandemic hoax was used as the excuse to issue almost limitless numbers of mail-in ballots with no checks to establish that the recipients were still alive or lived at that address. They were sent to streams of people who had not even asked for them. Private organisations were employed to gather these ballots and who knows what they did with them before they turned up at the counts. The American election system has been manipulated over decades to become a sick joke with more holes than a Swiss cheese for the express purpose of dictating the results. Then there was the criminal manipulation of information by Sabbatian tech giants like Facebook, Twitter and Google-owned YouTube which deleted pro-Trump, anti-Biden accounts and posts while everything in support of Biden was left alone. Sabbatians wanted Biden to win because after the dividing of America it was time for full-on Woke and every aspect of the Cult agenda to be unleashed.

Hunter gatherer

Extreme Silicon Valley bias included blocking information by the *New York Post* exposing a Biden scandal that should have ended his bid for president in the final weeks of the campaign. Hunter Biden, his monumentally corrupt son, is reported to have sent a laptop to be repaired at a local store and failed to return for it. Time passed until the laptop became the property of the store for non-payment of the bill. When the owner saw what was on the hard drive he gave a copy to the FBI who did nothing even though it confirmed widespread corruption in which the Joe Biden family were using his political position, especially when he was vice president to Obama, to make multiple millions in countries around the world and most notably Ukraine and China. Hunter Biden's one-time business partner Tony Bobulinski went public when the story broke in the *New York Post* to confirm the corruption he saw and that Joe Biden not only knew what was going on he also profited from the spoils. Millions were handed over by a Chinese company with close

connections – like all major businesses in China – to the Chinese communist party of President Xi Jinping. Joe Biden even boasted at a meeting of the Cult's World Economic Forum that as vice president he had ordered the government of Ukraine to fire a prosecutor. What he didn't mention was that the same man just happened to be investigating an energy company which was part of Hunter Biden's corrupt portfolio. The company was paying him big bucks for no other reason than the influence his father had. Overnight Biden's presidential campaign should have been over given that he had lied publicly about not knowing what his son was doing. Instead almost the entire Sabbatian-owned mainstream media and Sabbatian-owned Silicon Valley suppressed circulation of the story. This alone went a mighty way to rigging the election of 2020. Cult assets like Mark Zuckerberg at Facebook also spent hundreds of millions to be used in support of Biden and vote 'administration'.

The Cult had used Trump as the focus to divide America and was now desperate to bring in moronic, pliable, corrupt Biden to complete the double-whammy. No way were they going to let little things like the will of the people thwart their plan. Silicon Valley widely censored claims that the election was rigged because it *was* rigged. For the same reason anyone claiming it was rigged was denounced as a 'white supremacist' including the pathetically few Republican politicians willing to say so. Right across the media where the claim was mentioned it was described as a 'false claim' even though these excuses for 'journalists' would have done no research into the subject whatsoever. Trump won seven million more votes than any sitting president had ever achieved while somehow a cognitively-challenged soon to be 78-year-old who was hidden away from the public for most of the campaign managed to win more votes than any presidential candidate in history. It makes no sense. You only had to see election rallies for both candidates to witness the enthusiasm for Trump and the apathy for Biden. Tens of thousands would attend Trump events while Biden was speaking in empty car parks with often only television crews attending and framing their shots to hide the fact that no one was there. It was pathetic to see

footage come to light of Biden standing at a podium making speeches only to TV crews and party fixers while reading the words written for him on massive Teleprompter screens. So, yes, those protestors on January 6th had a point about election rigging, but some were about to walk into a trap laid for them in Washington by the Cult Deep State and its QAnon Psyop. This was the Capitol Hill riot ludicrously dubbed an 'insurrection'.

The spider and the fly

Renegade Minds know there are not two 'sides' in politics, only one side, the Cult, working through all 'sides'. It's a stage show, a puppet show, to direct the perceptions of the population into focusing on diversions like parties and candidates while missing the puppeteers with their hands holding all the strings. The Capitol Hill 'insurrection' brings us back to the Little Big Horn. Having created two distinct opposing groupings – Woke and Pushbackers – the trap was about to be sprung. Pushbackers were to be encircled and isolated by associating them all in the public mind with Trump and then labelling Trump as some sort of Confederate leader. I knew immediately that the Capitol riot was a set-up because of two things. One was how easy the rioters got into the building with virtually no credible resistance and secondly I could see – as with the 'Covid' hoax in the West at the start of 2020 – how the Cult could exploit the situation to move its agenda forward with great speed. My experience of Cult techniques and activities over more than 30 years has showed me that while they do exploit situations they haven't themselves created this never happens with events of fundamental agenda significance. Every time major events giving cultists the excuse to rapidly advance their plan you find they are manipulated into being for the specific reason of providing that excuse – Problem-Reaction-Solution. Only a tiny minority of the huge crowd of Washington protestors sought to gain entry to the Capitol by smashing windows and breaching doors. That didn't matter. The whole crowd and all Pushbackers, even if they did not support Trump, were going to be lumped together as dangerous

insurrectionists and conspiracy theorists. The latter term came into widespread use through a CIA memo in the 1960s aimed at discrediting those questioning the nonsensical official story of the Kennedy assassination and it subsequently became widely employed by the media. It's still being used by inept 'journalists' with no idea of its origin to discredit anyone questioning anything that authority claims to be true. When you are perpetrating a conspiracy you need to discredit the very word itself even though the dictionary definition of conspiracy is merely 'the activity of secretly planning with other people to do something bad or illegal' and 'a general agreement to keep silent about a subject for the purpose of keeping it secret'. On that basis there are conspiracies almost wherever you look. For obvious reasons the Cult and its lapdog media have to claim there are no conspiracies even though the word appears in state laws as with conspiracy to defraud, to murder, and to corrupt public morals.

Agent provocateurs are widely used by the Cult Deep State to manipulate genuine people into acting in ways that suit the desired outcome. By genuine in this case I mean protestors genuinely supporting Trump and claims that the election was stolen. In among them, however, were agents of the state wearing the garb of Trump supporters and QAnon to pump-prime the Capital riot which some genuine Trump supporters naively fell for. I described the situation as 'Come into my parlour said the spider to the fly'. Leaflets appeared through the Woke paramilitary arm Antifa, the anti-fascist fascists, calling on supporters to turn up in Washington looking like Trump supporters even though they hated him. Some of those arrested for breaching the Capitol Building were sourced to Antifa and its stable mate Black Lives Matter. Both organisations are funded by Cult billionaires and corporations. One man charged for the riot was according to his lawyer a former FBI agent who had held top secret security clearance for 40 years. Attorney Thomas Plofchan said of his client, 66-year-old Thomas Edward Caldwell:

He has held a Top Secret Security Clearance since 1979 and has undergone multiple Special Background Investigations in support of his clearances. After retiring from the Navy, he

worked as a section chief for the Federal Bureau of Investigation from 2009-2010 as a GS-12 [mid-level employee].

He also formed and operated a consulting firm performing work, often classified, for U.S government customers including the US. Drug Enforcement Agency, Department of Housing and Urban Development, the US Coast Guard, and the US Army Personnel Command.

A judge later released Caldwell pending trial in the absence of evidence about a conspiracy or that he tried to force his way into the building. *The New York Post* reported a 'law enforcement source' as saying that 'at least two known Antifa members were spotted' on camera among Trump supporters during the riot while one of the rioters arrested was John Earle Sullivan, a seriously extreme Black Lives Matter Trump-hater from Utah who was previously arrested and charged in July, 2020, over a BLM-Antifa riot in which drivers were threatened and one was shot. Sullivan is the founder of Utah-based Insurgence USA which is an affiliate of the Cult-created-and-funded Black Lives Matter movement. Footage appeared and was then deleted by Twitter of Trump supporters calling out Antifa infiltrators and a group was filmed changing into pro-Trump clothing before the riot. Security at the building was *pathetic* – as planned. Colonel Leroy Fletcher Prouty, a man with long experience in covert operations working with the US security apparatus, once described the tell-tale sign to identify who is involved in an assassination. He said:

No one has to direct an assassination – it happens. The active role is played secretly by permitting it to happen. This is the greatest single clue. Who has the power to call off or reduce the usual security precautions?

This principle applies to many other situations and certainly to the Capitol riot of January 6th, 2021.

The sting

With such a big and potentially angry crowd known to be gathering near the Capitol the security apparatus would have had a major police detail to defend the building with National Guard troops on

standby given the strength of feeling among people arriving from all over America encouraged by the QAnon Psyop and statements by Donald Trump. Instead Capitol Police 'security' was flimsy, weak, and easily breached. The same number of officers was deployed as on a regular day and that is a blatant red flag. They were not staffed or equipped for a possible riot that had been an obvious possibility in the circumstances. No protective and effective fencing worth the name was put in place and there were no contingency plans. The whole thing was basically a case of standing aside and waving people in. Once inside police mostly backed off apart from one Capitol police officer who ridiculously shot dead unarmed Air Force veteran protestor Ashli Babbitt without a warning as she climbed through a broken window. The 'investigation' refused to name or charge the officer after what must surely be considered a murder in the circumstances. They just lifted a carpet and swept. The story was endlessly repeated about five people dying in the 'armed insurrection' when there was no report of rioters using weapons. Apart from Babbitt the other four died from a heart attack, strokes and apparently a drug overdose. Capitol police officer Brian Sicknick was reported to have died after being bludgeoned with a fire extinguisher when he was alive after the riot was over and died later of what the Washington Medical Examiner's Office said was a stroke. Sicknick had no external injuries. The lies were delivered like rapid fire. There was a narrative to build with incessant repetition of the lie until the lie became the accepted 'everybody knows that' truth. The 'Big Lie' technique of Nazi Propaganda Minister Joseph Goebbels is constantly used by the Cult which was behind the Nazis and is today behind the 'Covid' and 'climate change' hoaxes. Goebbels said:

If you tell a lie big enough and keep repeating it, people will eventually come to believe it. The lie can be maintained only for such time as the State can shield the people from the political, economic and/or military consequences of the lie. It thus becomes vitally important for the State to use all of its powers to repress dissent, for the truth is the mortal enemy of the lie, and thus by extension, the truth is the greatest enemy of the State.

Most protestors had a free run of the Capitol Building. This allowed pictures to be taken of rioters in iconic parts of the building including the Senate chamber which could be used as propaganda images against all Pushbackers. One Congresswoman described the scene as 'the worst kind of non-security anybody could ever imagine'. Well, the first part was true, but someone obviously did imagine it and made sure it happened. Some photographs most widely circulated featured people wearing QAnon symbols and now the Psyop would be used to dub all QAnon followers with the ubiquitous fit-all label of 'white supremacist' and 'insurrectionists'. When a Muslim extremist called Noah Green drove his car at two police officers at the Capitol Building killing one in April, 2021, there was no such political and media hysteria. They were just disappointed he wasn't white.

The witch-hunt

Government prosecutor Michael Sherwin, an aggressive, dark-eyed, professional Rottweiler led the 'investigation' and to call it over the top would be to understate reality a thousand fold. Hundreds were tracked down and arrested for the crime of having the wrong political views and people were jailed who had done nothing more than walk in the building, committed no violence or damage to property, took a few pictures and left. They were labelled a 'threat to the Republic' while Biden sat in the White House signing executive orders written for him that were dismantling 'the Republic'. Even when judges ruled that a mother and son should not be in jail the government kept them there. Some of those arrested have been badly beaten by prison guards in Washington and lawyers for one man said he suffered a fractured skull and was made blind in one eye. Meanwhile a woman is shot dead for no reason by a Capitol Police officer and we are not allowed to know who he is never mind what has happened to him although that will be *nothing*. The Cult's QAnon/Trump sting to identify and isolate Pushbackers and then target them on the road to crushing and deleting them was a resounding success. You would have thought the Russians had

invaded the building at gunpoint and lined up senators for a firing squad to see the political and media reaction. Congresswoman Alexandria Ocasio-Cortez is a child in a woman's body, a terrible-twos, me, me, me, Woker narcissist of such proportions that words have no meaning. She said she thought she was going to die when 'insurrectionists' banged on her office door. It turned out she wasn't even in the Capitol Building when the riot was happening and the 'banging' was a Capitol Police officer. She referred to herself as a 'survivor' which is an insult to all those true survivors of violent and sexual abuse while she lives her pampered and privileged life talking drivel for a living. Her Woke colleague and fellow mega-narcissist Rashida Tlaib broke down describing the devastating effect on her, too, of *not being* in the building when the rioters were there. Ocasio-Cortez and Tlaib are members of a fully-Woke group of Congresswomen known as 'The Squad' along with Ilhan Omar and Ayanna Pressley. The Squad from what I can see can be identified by its vehement anti-white racism, anti-white men agenda, and, as always in these cases, the absence of brain cells on active duty.

The usual suspects were on the riot case immediately in the form of Democrat ultra-Zionist senators and operatives Chuck Schumer and Adam Schiff demanding that Trump be impeached for 'his part in the insurrection'. The same pair of prats had led the failed impeachment of Trump over the invented 'Russia collusion' nonsense which claimed Russia had helped Trump win the 2016 election. I didn't realise that Tel Aviv had been relocated just outside Moscow. I must find an up-to-date map. The Russia hoax was a Sabbatian operation to keep Trump occupied and impotent and to stop any rapport with Russia which the Cult wants to retain as a perceptual enemy to be pulled out at will. Puppet Biden began attacking Russia when he came to office as the Cult seeks more upheaval, division and war across the world. A two-year stage show 'Russia collusion inquiry' headed by the not-very-bright former 9/11 FBI chief Robert Mueller, with support from 19 lawyers, 40 FBI agents plus intelligence analysts, forensic accountants and other

staff, devoured tens of millions of dollars and found no evidence of Russia collusion which a ten-year-old could have told them on day one. Now the same moronic Schumer and Schiff wanted a second impeachment of Trump over the Capitol 'insurrection' (riot) which the arrested development of Schumer called another 'Pearl Harbor' while others compared it with 9/11 in which 3,000 died and, in the case of CNN, with the Rwandan genocide in the 1990s in which an estimated 500,000 to 600,000 were murdered, between 250, 000 and 500,000 women were raped, and populations of whole towns were hacked to death with machetes. To make those comparisons purely for Cult political reasons is beyond insulting to those that suffered and lost their lives and confirms yet again the callous inhumanity that we are dealing with. Schumer is a monumental idiot and so is Schiff, but they serve the Cult agenda and do whatever they're told so they get looked after. Talking of idiots – another inane man who spanned the Russia and Capitol impeachment attempts was Senator Eric Swalwell who had the nerve to accuse Trump of collusion with the Russians while sleeping with a Chinese spy called Christine Fang or 'Fang Fang' which is straight out of a Bond film no doubt starring Klaus Schwab as the bloke living on a secret island and controlling laser weapons positioned in space and pointing at world capitals. Fang Fang plays the part of Bond's infiltrator girlfriend which I'm sure she would enjoy rather more than sharing a bed with the brainless Swalwell, lying back and thinking of China. The FBI eventually warned Swalwell about Fang Fang which gave her time to escape back to the Chinese dictatorship. How very thoughtful of them. The second Trump impeachment also failed and hardly surprising when an impeachment is supposed to remove a sitting president and by the time it happened Trump was no longer president. These people are running your country America, well, officially anyway. Terrifying isn't it?

Outcomes tell the story - always

The outcome of all this – and it's the *outcome* on which Renegade Minds focus, not the words – was that a vicious, hysterical and

obviously pre-planned assault was launched on Pushbackers to censor, silence and discredit them and even targeted their right to earn a living. They have since been condemned as 'domestic terrorists' that need to be treated like Al-Qaeda and Islamic State. 'Domestic terrorists' is a label the Cult has been trying to make stick since the period of the Oklahoma bombing in 1995 which was blamed on 'far-right domestic terrorists'. If you read *The Trigger* you will see that the bombing was clearly a Problem-Reaction-Solution carried out by the Deep State during a Bill Clinton administration so corrupt that no dictionary definition of the term would even nearly suffice. Nearly 30, 000 troops were deployed from all over America to the empty streets of Washington for Biden's inauguration. Ten thousand of them stayed on with the pretext of protecting the capital from insurrectionists when it was more psychological programming to normalise the use of the military in domestic law enforcement in support of the Cult plan for a police-military state. Biden's fascist administration began a purge of 'wrong-thinkers' in the military which means anyone that is not on board with Woke. The Capitol Building was surrounded by a fence with razor wire and the Land of the Free was further symbolically and literally dismantled. The circle was completed with the installation of Biden and the exploitation of the QAnon Psyop.

America had never been so divided since the civil war of the 19th century, Pushbackers were isolated and dubbed terrorists and now, as was always going to happen, the Cult immediately set about deleting what little was left of freedom and transforming American society through a swish of the hand of the most controlled 'president' in American history leading (officially at least) the most extreme regime since the country was declared an independent state on July 4th, 1776. Biden issued undebated, dictatorial executive orders almost by the hour in his opening days in office across the whole spectrum of the Cult wish-list including diluting controls on the border with Mexico allowing thousands of migrants to illegally enter the United States to transform the demographics of America and import an election-changing number of perceived Democrat

voters. Then there were Biden deportation amnesties for the already illegally resident (estimated to be as high as 20 or even 30 million). A bill before Congress awarded American citizenship to anyone who could prove they had worked in agriculture for just 180 days in the previous two years as 'Big Ag' secured its slave labour long-term. There were the plans to add new states to the union such as Puerto Rico and making Washington DC a state. They are all parts of a plan to ensure that the Cult-owned Woke Democrats would be permanently in power.

Border – what border?

I have exposed in detail in other books how mass immigration into the United States and Europe is the work of Cult networks fuelled by the tens of billions spent to this and other ends by George Soros and his global Open Society (open borders) Foundations. The impact can be seen in America alone where the population has increased by *100 million* in little more than 30 years mostly through immigration. I wrote in *The Answer* that the plan was to have so many people crossing the southern border that the numbers become unstoppable and we are now there under Cult-owned Biden. El Salvador in Central America puts the scale of what is happening into context. A third of the population now lives in the United States, much of it illegally, and many more are on the way. The methodology is to crush Central and South American countries economically and spread violence through machete-wielding psychopathic gangs like MS-13 based in El Salvador and now operating in many American cities. Biden-imposed lax security at the southern border means that it is all but open. He said before his 'election' that he wanted to see a surge towards the border if he became president and that was the green light for people to do just that after election day to create the human disaster that followed for both America and the migrants. When that surge came the imbecilic Alexandria Ocasio-Cortez said it wasn't a 'surge' because they are 'children, not insurgents' and the term 'surge' (used by Biden) was a claim of 'white supremacists'.

This disingenuous lady may one day enter the realm of the most basic intelligence, but it won't be any time soon.

Sabbatians and the Cult are in the process of destroying America by importing violent people and gangs in among the genuine to terrorise American cities and by overwhelming services that cannot cope with the sheer volume of new arrivals. Something similar is happening in Europe as Western society in general is targeted for demographic and cultural transformation and upheaval. The plan demands violence and crime to create an environment of intimidation, fear and division and Soros has been funding the election of district attorneys across America who then stop prosecuting many crimes, reduce sentences for violent crimes and free as many violent criminals as they can. Sabbatians are creating the chaos from which order – their order – can respond in a classic Problem-Reaction-Solution. A Freemasonic moto says 'Ordo Ab Chao' (Order out of Chaos) and this is why the Cult is constantly creating chaos to impose a new 'order'. Here you have the reason the Cult is constantly creating chaos. The 'Covid' hoax can be seen with those entering the United States by plane being forced to take a 'Covid' test while migrants flooding through southern border processing facilities do not. Nothing is put in the way of mass migration and if that means ignoring the government's own 'Covid' rules then so be it. They know it's all bullshit anyway. Any pushback on this is denounced as 'racist' by Wokers and Sabbatian fronts like the ultra-Zionist Anti-Defamation League headed by the appalling Jonathan Greenblatt which at the same time argues that Israel should not give citizenship and voting rights to more Palestinian Arabs or the 'Jewish population' (in truth the Sabbatian network) will lose control of the country.

Society-changing numbers

Biden's masters have declared that countries like El Salvador are so dangerous that their people must be allowed into the United States for humanitarian reasons when there are fewer murders in large parts of many Central American countries than in US cities like

Baltimore. That is not to say Central America cannot be a dangerous place and Cult-controlled American governments have been making it so since way back, along with the dismantling of economies, in a long-term plan to drive people north into the United States. Parts of Central America are very dangerous, but in other areas the story is being greatly exaggerated to justify relaxing immigration criteria. Migrants are being offered free healthcare and education in the United States as another incentive to head for the border and there is no requirement to be financially independent before you can enter to prevent the resources of America being drained. You can't blame migrants for seeking what they believe will be a better life, but they are being played by the Cult for dark and nefarious ends. The numbers since Biden took office are huge. In February, 2021, more than 100,000 people were known to have tried to enter the US illegally through the southern border (it was 34,000 in the same month in 2020) and in March it was 170,000 – a 418 percent increase on March, 2020. These numbers are only known people, not the ones who get in unseen. The true figure for migrants illegally crossing the border in a single month was estimated by one congressman at 250,000 and that number will only rise under Biden's current policy. Gangs of murdering drug-running thugs that control the Mexican side of the border demand money – thousands of dollars – to let migrants cross the Rio Grande into America. At the same time gun battles are breaking out on the border several times a week between rival Mexican drug gangs (which now operate globally) who are equipped with sophisticated military-grade weapons, grenades and armoured vehicles. While the Capitol Building was being 'protected' from a non-existent 'threat' by thousands of troops, and others were still deployed at the time in the Cult Neocon war in Afghanistan, the southern border of America was left to its fate. This is not incompetence, it is cold calculation.

By March, 2021, there were 17,000 unaccompanied children held at border facilities and many of them are ensnared by people traffickers for paedophile rings and raped on their journey north to America. This is not conjecture – this is fact. Many of those designated

children are in reality teenage boys or older. Meanwhile Wokers posture their self-purity for encouraging poor and tragic people to come to America and face this nightmare both on the journey and at the border with the disgusting figure of House Speaker Nancy Pelosi giving disingenuous speeches about caring for migrants. The woman's evil. Wokers condemned Trump for having children in cages at the border (so did Obama, *Shhhh*), but now they are sleeping on the floor without access to a shower with one border facility 729 percent over capacity. The Biden insanity even proposed flying migrants from the southern border to the northern border with Canada for 'processing'. The whole shambles is being overseen by ultra-Zionist Secretary of Homeland Security, the moronic liar Alejandro Mayorkas, who banned news cameras at border facilities to stop Americans seeing what was happening. Mayorkas said there was not a ban on news crews; it was just that they were not allowed to film. Alongside him at Homeland Security is another ultra-Zionist Cass Sunstein appointed by Biden to oversee new immigration laws. Sunstein despises conspiracy researchers to the point where he suggests they should be banned or *taxed* for having such views. The man is not bonkers or anything. He's perfectly well-adjusted, but adjusted to what is the question. Criticise what is happening and you are a 'white supremacist' when earlier non-white immigrants also oppose the numbers which effect their lives and opportunities. Black people in poor areas are particularly damaged by uncontrolled immigration and the increased competition for work opportunities with those who will work for less. They are also losing voting power as Hispanics become more dominant in former black areas. It's a downward spiral for them while the billionaires behind the policy drone on about how much they care about black people and 'racism'. None of this is about compassion for migrants or black people – that's just wind and air. Migrants are instead being mercilessly exploited to transform America while the countries they leave are losing their future and the same is true in Europe. Mass immigration may now be the work of Woke Democrats, but it can be traced back to the 1986 Immigration Reform and Control Act (it

wasn't) signed into law by Republican hero President Ronald Reagan which gave amnesty to millions living in the United States illegally and other incentives for people to head for the southern border. Here we have the one-party state at work again.

Save me syndrome

Almost every aspect of what I have been exposing as the Cult agenda was on display in even the first days of 'Biden' with silencing of Pushbackers at the forefront of everything. A Renegade Mind will view the Trump years and QAnon in a very different light to their supporters and advocates as the dots are connected. The QAnon/Trump Psyop has given the Cult all it was looking for. We may not know how much, or little, that Trump realised he was being used, but that's a side issue. This pincer movement produced the desired outcome of dividing America and having Pushbackers isolated. To turn this around we have to look at new routes to empowerment which do not include handing our power to other people and groups through what I will call the 'Save Me Syndrome' – 'I want someone else to do it so that I don't have to'. We have seen this at work throughout human history and the QAnon/Trump Psyop is only the latest incarnation alongside all the others. Religion is an obvious expression of this when people look to a 'god' or priest to save them or tell them how to be saved and then there are 'save me' politicians like Trump. Politics is a diversion and not a 'saviour'. It is a means to block positive change, not make it possible.

Save Me Syndrome always comes with the same repeating theme of handing your power to whom or what you believe will save you while your real 'saviour' stares back from the mirror every morning. Renegade Minds are constantly vigilant in this regard and always asking the question 'What can I do?' rather than 'What can someone else do for me?' Gandhi was right when he said: 'You must be the change you want to see in the world.' We are indeed the people we have been waiting for. We are presented with a constant raft of reasons to concede that power to others and forget where the real power is. Humanity has the numbers and the Cult does not. It has to

use diversion and division to target the unstoppable power that comes from unity. Religions, governments, politicians, corporations, media, QAnon, are all different manifestations of this power-diversion and dilution. Refusing to give your power to governments and instead handing it to Trump and QAnon is not to take a new direction, but merely to recycle the old one with new names on the posters. I will explore this phenomenon as we proceed and how to break the cycles and recycles that got us here through the mists of repeating perception and so repeating history.

For now we shall turn to the most potent example in the entire human story of the consequences that follow when you give your power away. I am talking, of course, of the 'Covid' hoax.

CHAPTER FOUR

'Covid': Calculated catastrophe

Facts are threatening to those invested in fraud
DaShanne Stokes

We can easily unravel the real reason for the 'Covid pandemic' hoax by employing the Renegade Mind methodology that I have outlined this far. We'll start by comparing the long-planned Cult outcome with the 'Covid pandemic' outcome. Know the outcome and you'll see the journey.

I have highlighted the plan for the Hunger Games Society which has been in my books for so many years with the very few controlling the very many through ongoing dependency. To create this dependency it is essential to destroy independent livelihoods, businesses and employment to make the population reliant on the state (the Cult) for even the basics of life through a guaranteed pittance income. While independence of income remained these Cult ambitions would be thwarted. With this knowledge it was easy to see where the 'pandemic' hoax was going once talk of 'lockdowns' began and the closing of all but perceived 'essential' businesses to 'save' us from an alleged 'deadly virus'. Cult corporations like Amazon and Walmart were naturally considered 'essential' while mom and pop shops and stores had their doors closed by fascist decree. As a result with every new lockdown and new regulation more small and medium, even large businesses not owned by the Cult, went to the wall while Cult giants and their frontmen and women grew financially fatter by the second. Mom and pop were

denied an income and the right to earn a living and the wealth of people like Jeff Bezos (Amazon), Mark Zuckerberg (Facebook) and Sergei Brin and Larry Page (Google/Alphabet) have reached record levels. The Cult was increasing its own power through further dramatic concentrations of wealth while the competition was being destroyed and brought into a state of dependency. Lockdowns have been instigated to secure that very end and were never anything to do with health. My brother Paul spent 45 years building up a bus repair business, but lockdowns meant buses were running at a fraction of normal levels for months on end. Similar stories can told in their hundreds of millions worldwide. Efforts of a lifetime coldly destroyed by Cult multi-billionaires and their lackeys in government and law enforcement who continued to earn their living from the taxation of the people while denying the right of the same people to earn theirs. How different it would have been if those making and enforcing these decisions had to face the same financial hardships of those they affected, but they never do.

Gates of Hell

Behind it all in the full knowledge of what he is doing and why is the psychopathic figure of Cult operative Bill Gates. His puppet Tedros at the World Health Organization declared 'Covid' a pandemic in March, 2020. The WHO had changed the definition of a 'pandemic' in 2009 just a month before declaring the 'swine flu pandemic' which would not have been so under the previous definition. The same applies to 'Covid'. The definition had included… 'an infection by an infectious agent, occurring simultaneously in different countries, with a significant mortality rate relative to the proportion of the population infected'. The new definition removed the need for 'significant mortality'. The 'pandemic' has been fraudulent even down to the definition, but Gates demanded economy-destroying lockdowns, school closures, social distancing, mandatory masks, a 'vaccination' for every man, woman and child on the planet and severe consequences and restrictions for those that refused. Who gave him this power? The

Cult did which he serves like a little boy in short trousers doing what his daddy tells him. He and his psychopathic missus even smiled when they said that much worse was to come (what they knew was planned to come). Gates responded in the matter-of-fact way of all psychopaths to a question about the effect on the world economy of what he was doing:

> Well, it won't go to zero but it will shrink. Global GDP is probably going to take the biggest hit ever [Gates was smiling as he said this] … in my lifetime this will be the greatest economic hit. But you don't have a choice. People act as if you have a choice. People don't feel like going to the stadium when they might get infected … People are deeply affected by seeing these stats, by knowing they could be part of the transmission chain, old people, their parents and grandparents, could be affected by this, and so you don't get to say ignore what is going on here.
>
> There will be the ability to open up, particularly in rich countries, if things are done well over the next few months, but for the world at large normalcy only returns when we have largely vaccinated the entire population.

The man has no compassion or empathy. How could he when he's a psychopath like all Cult players? My own view is that even beyond that he is very seriously mentally ill. Look in his eyes and you can see this along with his crazy flailing arms. You don't do what he has done to the world population since the start of 2020 unless you are mentally ill and at the most extreme end of psychopathic. You especially don't do it when to you know, as we shall see, that cases and deaths from 'Covid' are fakery and a product of monumental figure massaging. 'These stats' that Gates referred to are based on a 'test' that's not testing for the 'virus' as he has known all along. He made his fortune with big Cult support as an infamously ruthless software salesman and now buys global control of 'health' (death) policy without the population he affects having any say. It's a breathtaking outrage. Gates talked about people being deeply affected by fear of 'Covid' when that was because of *him* and his global network lying to them minute-by-minute supported by a lying media that he seriously influences and funds to the tune of hundreds of millions. He's handed big sums to media operations including the BBC, NBC, Al Jazeera, Univision, *PBS NewsHour*,

ProPublica, National Journal, The Guardian, The Financial Times, The Atlantic, Texas Tribune, USA Today publisher Gannett, *Washington Monthly, Le Monde,* Center for Investigative Reporting, Pulitzer Center on Crisis Reporting, National Press Foundation, International Center for Journalists, Solutions Journalism Network, the Poynter Institute for Media Studies, and many more. Gates is everywhere in the 'Covid' hoax and the man must go to prison – or a mental facility – for the rest of his life and his money distributed to those he has taken such enormous psychopathic pleasure in crushing.

The Muscle

The Hunger Games global structure demands a police-military state – a fusion of the two into one force – which viciously imposes the will of the Cult on the population and protects the Cult from public rebellion. In that regard, too, the 'Covid' hoax just keeps on giving. Often unlawful, ridiculous and contradictory 'Covid' rules and regulations have been policed across the world by moronic automatons and psychopaths made faceless by face-nappy masks and acting like the Nazi SS and fascist blackshirts and brownshirts of Hitler and Mussolini. The smallest departure from the rules decreed by the psychos in government and their clueless gofers were jumped upon by the face-nappy fascists. Brutality against public protestors soon became commonplace even on girls, women and old people as the brave men with the batons – the Face-Nappies as I call them – broke up peaceful protests and handed out fines like confetti to people who couldn't earn a living let alone pay hundreds of pounds for what was once an accepted human right. Robot Face-Nappies of Nottingham police in the English East Midlands fined one group £11,000 for attending a child's birthday party. For decades I charted the transformation of law enforcement as genuine, decent officers were replaced with psychopaths and the brain dead who would happily and brutally do whatever their masters told them. Now they were let loose on the public and I would emphasise the point that none of this just happened. The step-by-step change in the dynamic between police and public was orchestrated from the shadows by

those who knew where this was all going and the same with the perceptual reframing of those in all levels of authority and official administration through 'training courses' by organisations such as Common Purpose which was created in the late 1980s and given a massive boost in Blair era Britain until it became a global phenomenon. Supposed public 'servants' began to view the population as the enemy and the same was true of the police. This was the start of the explosion of behaviour manipulation organisations and networks preparing for the all-war on the human psyche unleashed with the dawn of 2020. I will go into more detail about this later in the book because it is a core part of what is happening.

Police desecrated beauty spots to deter people gathering and arrested women for walking in the countryside alone 'too far' from their homes. We had arrogant, clueless sergeants in the Isle of Wight police where I live posting on Facebook what they insisted the population must do or else. A schoolmaster sergeant called Radford looked young enough for me to ask if his mother knew he was out, but he was posting what he *expected* people to do while a Sergeant Wilkinson boasted about fining lads for meeting in a McDonald's car park where they went to get a lockdown takeaway. Wilkinson added that he had even cancelled their order. What a pair of prats these people are and yet they have increasingly become the norm among Jackboot Johnson's Yellowshirts once known as the British police. This was the theme all over the world with police savagery common during lockdown protests in the United States, the Netherlands, and the fascist state of Victoria in Australia under its tyrannical and again moronic premier Daniel Andrews. Amazing how tyrannical and moronic tend to work as a team and the same combination could be seen across America as arrogant, narcissistic Woke governors and mayors such as Gavin Newsom (California), Andrew Cuomo (New York), Gretchen Whitmer (Michigan), Lori Lightfoot (Chicago) and Eric Garcetti (Los Angeles) did their Nazi and Stalin impressions with the full support of the compliant brutality of their enforcers in uniform as they arrested small business owners defying

fascist shutdown orders and took them to jail in ankle shackles and handcuffs. This happened to bistro owner Marlena Pavlos-Hackney in Gretchen Whitmer's fascist state of Michigan when police arrived to enforce an order by a state-owned judge for 'putting the community at risk' at a time when other states like Texas were dropping restrictions and migrants were pouring across the southern border without any 'Covid' questions at all. I'm sure there are many officers appalled by what they are ordered to do, but not nearly enough of them. If they were truly appalled they would not do it. As the months passed every opportunity was taken to have the military involved to make their presence on the streets ever more familiar and 'normal' for the longer-term goal of police-military fusion.

Another crucial element to the Hunger Games enforcement network has been encouraging the public to report neighbours and others for 'breaking the lockdown rules'. The group faced with £11,000 in fines at the child's birthday party would have been dobbed-in by a neighbour with a brain the size of a pea. The technique was most famously employed by the Stasi secret police in communist East Germany who had public informants placed throughout the population. A police chief in the UK says his force doesn't need to carry out 'Covid' patrols when they are flooded with so many calls from the public reporting other people for visiting the beach. Dorset police chief James Vaughan said people were so enthusiastic about snitching on their fellow humans they were now operating as an auxiliary arm of the police: 'We are still getting around 400 reports a week from the public, so we will respond to reports …We won't need to be doing hotspot patrols because people are very quick to pick the phone up and tell us.' Vaughan didn't say that this is a pillar of all tyrannies of whatever complexion and the means to hugely extend the reach of enforcement while spreading distrust among the people and making them wary of doing anything that might get them reported. Those narcissistic Isle of Wight sergeants Radford and Wilkinson never fail to add a link to their Facebook posts where the public can inform on their fellow slaves.

Neither would be self-aware enough to realise they were imitating the Stasi which they might well never have heard of. Government psychologists that I will expose later laid out a policy to turn communities against each other in the same way.

A coincidence? Yep, and I can knit fog

I knew from the start of the alleged pandemic that this was a Cult operation. It presented limitless potential to rapidly advance the Cult agenda and exploit manipulated fear to demand that every man, woman and child on the planet was 'vaccinated' in a process never used on humans before which infuses self-replicating *synthetic* material into human cells. Remember the plan to transform the human body from a biological to a synthetic biological state. I'll deal with the 'vaccine' (that's not actually a vaccine) when I focus on the genetic agenda. Enough to say here that mass global 'vaccination' justified by this 'new virus' set alarms ringing after 30 years of tracking these people and their methods. The 'Covid' hoax officially beginning in China was also a big red flag for reasons I will be explaining. The agenda potential was so enormous that I could dismiss any idea that the 'virus' appeared naturally. Major happenings with major agenda implications never occur without Cult involvement in making them happen. My questions were twofold in early 2020 as the media began its campaign to induce global fear and hysteria: Was this alleged infectious agent released on purpose by the Cult or did it even exist at all? I then did what I always do in these situations. I sat, observed and waited to see where the evidence and information would take me. By March and early April synchronicity was strongly – and ever more so since then – pointing me in the direction of *there is no 'virus'*. I went public on that with derision even from swathes of the alternative media that voiced a scenario that the Chinese government released the 'virus' in league with Deep State elements in the United States from a top-level bio-lab in Wuhan where the 'virus' is said to have first appeared. I looked at that possibility, but I didn't buy it for several reasons. Deaths from the 'virus' did not in any way match what they

would have been with a 'deadly bioweapon' and it is much more effective if you sell the *illusion* of an infectious agent rather than having a real one unless you can control through injection who has it and who doesn't. Otherwise you lose control of events. A made-up 'virus' gives you a blank sheet of paper on which you can make it do whatever you like and have any symptoms or mutant 'variants' you choose to add while a real infectious agent would limit you to what it actually does. A phantom disease allows you to have endless ludicrous 'studies' on the 'Covid' dollar to widen the perceived impact by inventing ever more 'at risk' groups including one study which said those who walk slowly may be almost four times more likely to die from the 'virus'. People are in psychiatric wards for less.

A real 'deadly bioweapon' can take out people in the hierarchy that are not part of the Cult, but essential to its operation. Obviously they don't want that. Releasing a real disease means you immediately lose control of it. Releasing an illusory one means you don't. Again it's vital that people are extra careful when dealing with what they want to hear. A bioweapon unleashed from a Chinese laboratory in collusion with the American Deep State may fit a conspiracy narrative, but is it true? Would it not be far more effective to use the excuse of a 'virus' to justify the real bioweapon – the 'vaccine'? That way your disease agent does not have to be transmitted and arrives directly through a syringe. I saw a French virologist Luc Montagnier quoted in the alternative media as saying he had discovered that the alleged 'new' severe acute respiratory syndrome coronavirus , or SARS-CoV-2, was made artificially and included elements of the human immunodeficiency 'virus' (HIV) and a parasite that causes malaria. SARS-CoV-2 is alleged to trigger an alleged illness called Covid-19. I remembered Montagnier's name from my research years before into claims that an HIV 'retrovirus' causes AIDs – claims that were demolished by Berkeley virologist Peter Duesberg who showed that no one had ever proved that HIV causes acquired immunodeficiency syndrome or AIDS. Claims that become accepted as fact, publicly and medically, with no proof whatsoever are an ever-recurring story that profoundly applies to

'Covid'. Nevertheless, despite the lack of proof, Montagnier's team at the Pasteur Institute in Paris had a long dispute with American researcher Robert Gallo over which of them discovered and isolated the HIV 'virus' and with *no evidence* found it to cause AIDS. You will see later that there is also no evidence that any 'virus' causes any disease or that there is even such a thing as a 'virus' in the way it is said to exist. The claim to have 'isolated' the HIV 'virus' will be presented in its real context as we come to the shocking story – and it is a story – of SARS-CoV-2 and so will Montagnier's assertion that he identified the full SARS-CoV-2 genome.

Hoax in the making

We can pick up the 'Covid' story in 2010 and the publication by the Rockefeller Foundation of a document called 'Scenarios for the Future of Technology and International Development'. The inner circle of the Rockefeller family has been serving the Cult since John D. Rockefeller (1839-1937) made his fortune with Standard Oil. It is less well known that the same Rockefeller – the Bill Gates of his day – was responsible for establishing what is now referred to as 'Big Pharma', the global network of pharmaceutical companies that make outrageous profits dispensing scalpel and drug 'medicine' and are obsessed with pumping vaccines in ever-increasing number into as many human arms and backsides as possible. John D. Rockefeller was the driving force behind the creation of the 'education' system in the United States and elsewhere specifically designed to program the perceptions of generations thereafter. The Rockefeller family donated exceptionally valuable land in New York for the United Nations building and were central in establishing the World Health Organization in 1948 as an agency of the UN which was created from the start as a Trojan horse and stalking horse for world government. Now enter Bill Gates. His family and the Rockefellers have long been extremely close and I have seen genealogy which claims that if you go back far enough the two families fuse into the same bloodline. Gates has said that the Bill and Melinda Gates Foundation was inspired by the Rockefeller Foundation and why not

when both are serving the same Cult? Major tax-exempt foundations are overwhelmingly criminal enterprises in which Cult assets fund the Cult agenda in the guise of 'philanthropy' while avoiding tax in the process. Cult operatives can become mega-rich in their role of front men and women for the psychopaths at the inner core and they, too, have to be psychopaths to knowingly serve such evil. Part of the deal is that a big percentage of the wealth gleaned from representing the Cult has to be spent advancing the ambitions of the Cult and hence you have the Rockefeller Foundation, Bill and Melinda Gates Foundation (and *so* many more) and people like George Soros with his global Open Society Foundations spending their billions in pursuit of global Cult control. Gates is a global public face of the Cult with his interventions in world affairs including Big Tech influence; a central role in the 'Covid' and 'vaccine' scam; promotion of the climate change shakedown; manipulation of education; geoengineering of the skies; and his food-control agenda as the biggest owner of farmland in America, his GMO promotion and through other means. As one writer said: 'Gates monopolizes or wields disproportionate influence over the tech industry, global health and vaccines, agriculture and food policy (including biopiracy and fake food), weather modification and other climate technologies, surveillance, education and media.' The almost limitless wealth secured through Microsoft and other not-allowed-to-fail ventures (including vaccines) has been ploughed into a long, long list of Cult projects designed to enslave the entire human race. Gates and the Rockefellers have been working as one unit with the Rockefeller-established World Health Organization leading global 'Covid' policy controlled by Gates through his mouth-piece Tedros. Gates became the WHO's biggest funder when Trump announced that the American government would cease its donations, but Biden immediately said he would restore the money when he took office in January, 2021. The Gates Foundation (the Cult) owns through limitless funding the world health system and the major players across the globe in the 'Covid' hoax.

Okay, with that background we return to that Rockefeller Foundation document of 2010 headed 'Scenarios for the Future of Technology and International Development' and its 'imaginary' epidemic of a virulent and deadly influenza strain which infected 20 percent of the global population and killed eight million in seven months. The Rockefeller scenario was that the epidemic destroyed economies, closed shops, offices and other businesses and led to governments imposing fierce rules and restrictions that included mandatory wearing of face masks and body-temperature checks to enter communal spaces like railway stations and supermarkets. The document predicted that even after the height of the Rockefeller-envisaged epidemic the authoritarian rule would continue to deal with further pandemics, transnational terrorism, environmental crises and rising poverty. Now you may think that the Rockefellers are our modern-day seers or alternatively, and rather more likely, that they well knew what was planned a few years further on. Fascism had to be imposed, you see, to 'protect citizens from risk and exposure'. The Rockefeller scenario document said:

During the pandemic, national leaders around the world flexed their authority and imposed airtight rules and restrictions, from the mandatory wearing of face masks to body-temperature checks at the entries to communal spaces like train stations and supermarkets. Even after the pandemic faded, this more authoritarian control and oversight of citizens and their activities stuck and even intensified. In order to protect themselves from the spread of increasingly global problems – from pandemics and transnational terrorism to environmental crises and rising poverty – leaders around the world took a firmer grip on power.

At first, the notion of a more controlled world gained wide acceptance and approval. Citizens willingly gave up some of their sovereignty – and their privacy – to more paternalistic states in exchange for greater safety and stability. Citizens were more tolerant, and even eager, for top-down direction and oversight, and national leaders had more latitude to impose order in the ways they saw fit.

In developed countries, this heightened oversight took many forms: biometric IDs for all citizens, for example, and tighter regulation of key industries whose stability was deemed vital to national interests. In many developed countries, enforced cooperation with a suite of new regulations and agreements slowly but steadily restored both order and, importantly, economic growth.

There we have the prophetic Rockefellers in 2010 and three years later came their paper for the Global Health Summit in Beijing, China, when government representatives, the private sector, international organisations and groups met to discuss the next 100 years of 'global health'. The Rockefeller Foundation-funded paper was called 'Dreaming the Future of Health for the Next 100 Years and more prophecy ensued as it described a dystopian future: 'The abundance of data, digitally tracking and linking people may mean the 'death of privacy' and may replace physical interaction with transient, virtual connection, generating isolation and raising questions of how values are shaped in virtual networks.' Next in the 'Covid' hoax preparation sequence came a 'table top' simulation in 2018 for another 'imaginary' pandemic of a disease called Clade X which was said to kill 900 million people. The exercise was organised by the Gates-funded Johns Hopkins University's Center for Health Security in the United States and this is the very same university that has been compiling the disgustingly and systematically erroneous global figures for 'Covid' cases and deaths. Similar Johns Hopkins health crisis scenarios have included the Dark Winter exercise in 2001 and Atlantic Storm in 2005.

Nostradamus 201

For sheer predictive genius look no further prophecy-watchers than the Bill Gates-funded Event 201 held only six weeks before the 'coronavirus pandemic' is supposed to have broken out in China and Event 201 was based on a scenario of a global 'coronavirus pandemic'. Melinda Gates, the great man's missus, told the BBC that he had 'prepared for years' for a coronavirus pandemic which told us what we already knew. Nostradamugates had predicted in a TED talk in 2015 that a pandemic was coming that would kill a lot of people and demolish the world economy. My god, the man is a machine – possibly even literally. Now here he was only weeks before the real thing funding just such a simulated scenario and involving his friends and associates at Johns Hopkins, the World Economic Forum Cult-front of Klaus Schwab, the United Nations,

Johnson & Johnson, major banks, and officials from China and the Centers for Disease Control in the United States. What synchronicity – Johns Hopkins would go on to compile the fraudulent 'Covid' figures, the World Economic Forum and Schwab would push the 'Great Reset' in response to 'Covid', the Centers for Disease Control would be at the forefront of 'Covid' policy in the United States, Johnson & Johnson would produce a 'Covid vaccine', and everything would officially start just weeks later in China. Spooky, eh? They were even accurate in creating a simulation of a 'virus' pandemic because the 'real thing' would also be a simulation. Event 201 was not an exercise preparing for something that might happen; it was a rehearsal for what those in control knew was *going* to happen and very shortly. Hours of this simulation were posted on the Internet and the various themes and responses mirrored what would soon be imposed to transform human society. News stories were inserted and what they said would be commonplace a few weeks later with still more prophecy perfection. Much discussion focused on the need to deal with misinformation and the 'anti-vax movement' which is exactly what happened when the 'virus' arrived – was said to have arrived – in the West.

Cult-owned social media banned criticism and exposure of the official 'virus' narrative and when I said there *was* no 'virus' in early April, 2020, I was banned by one platform after another including YouTube, Facebook and later Twitter. The mainstream broadcast media in Britain was in effect banned from interviewing me by the Tony-Blair-created government broadcasting censor Ofcom headed by career government bureaucrat Melanie Dawes who was appointed just as the 'virus' hoax was about to play out in January, 2020. At the same time the Ickonic media platform was using Vimeo, another ultra-Zionist-owned operation, while our own player was being created and they deleted in an instant hundreds of videos, documentaries, series and shows to confirm their unbelievable vindictiveness. We had copies, of course, and they had to be restored one by one when our player was ready. These people have no class. Sabbatian Facebook promised free advertisements for the Gates-

controlled World Health Organization narrative while deleting 'false claims and conspiracy theories' to stop 'misinformation' about the alleged coronavirus. All these responses could be seen just a short while earlier in the scenarios of Event 201. Extreme censorship was absolutely crucial for the Cult because the official story was so ridiculous and unsupportable by the evidence that it could never survive open debate and the free-flow of information and opinion. If you can't win a debate then don't have one is the Cult's approach throughout history. Facebook's little boy front man – front boy – Mark Zuckerberg equated 'credible and accurate information' with official sources and exposing their lies with 'misinformation'.

Silencing those that can see

The censorship dynamic of Event 201 is now the norm with an army of narrative-supporting 'fact-checker' organisations whose entire reason for being is to tell the public that official narratives are true and those exposing them are lying. One of the most appalling of these 'fact-checkers' is called NewsGuard founded by ultra-Zionist Americans Gordon Crovitz and Steven Brill. Crovitz is a former publisher of *The Wall Street Journal*, former Executive Vice President of Dow Jones, a member of the Council on Foreign Relations (CFR), and on the board of the American Association of Rhodes Scholars. The CFR and Rhodes Scholarships, named after Rothschild agent Cecil Rhodes who plundered the gold and diamonds of South Africa for his masters and the Cult, have featured widely in my books. NewsGuard don't seem to like me for some reason – I really can't think why – and they have done all they can to have me censored and discredited which is, to quote an old British politician, like being savaged by a dead sheep. They are, however, like all in the censorship network, very well connected and funded by organisations themselves funded by, or connected to, Bill Gates. As you would expect with anything associated with Gates NewsGuard has an offshoot called HealthGuard which 'fights online health care hoaxes'. How very kind. Somehow the NewsGuard European Managing Director Anna-Sophie Harling, a remarkably young-

looking woman with no broadcasting experience and little hands-on work in journalism, has somehow secured a position on the 'Content Board' of UK government broadcast censor Ofcom. An executive of an organisation seeking to discredit dissidents of the official narratives is making decisions for the government broadcast 'regulator' about content?? Another appalling 'fact-checker' is Full Fact funded by George Soros and global censors Google and Facebook.

It's amazing how many activists in the 'fact-checking', 'anti-hate', arena turn up in government-related positions – people like UK Labour Party activist Imran Ahmed who heads the Center for Countering Digital Hate founded by people like Morgan McSweeney, now chief of staff to the Labour Party's hapless and useless 'leader' Keir Starmer. Digital Hate – which is what it really is – uses the American spelling of Center to betray its connection to a transatlantic network of similar organisations which in 2020 shapeshifted from attacking people for 'hate' to attacking them for questioning the 'Covid' hoax and the dangers of the 'Covid vaccine'. It's just a coincidence, you understand. This is one of Imran Ahmed's hysterical statements: 'I would go beyond calling anti-vaxxers conspiracy theorists to say they are an extremist group that pose a national security risk.' No one could ever accuse this prat of understatement and he's including in that those parents who are now against vaccines after their children were damaged for life or killed by them. He's such a nice man. Ahmed does the rounds of the Woke media getting soft-ball questions from spineless 'journalists' who never ask what right he has to campaign to destroy the freedom of speech of others while he demands it for himself. There also seems to be an overrepresentation in Ofcom of people connected to the narrative-worshipping BBC. This incredible global network of narrative-support was super-vital when the 'Covid' hoax was played in the light of the mega-whopper lies that have to be defended from the spotlight cast by the most basic intelligence.

Setting the scene

The Cult plays the long game and proceeds step-by-step ensuring that everything is in place before major cards are played and they don't come any bigger than the 'Covid' hoax. The psychopaths can't handle events where the outcome isn't certain and as little as possible – preferably nothing – is left to chance. Politicians, government and medical officials who would follow direction were brought to illusory power in advance by the Cult web whether on the national stage or others like state governors and mayors of America. For decades the dynamic between officialdom, law enforcement and the public was changed from one of service to one of control and dictatorship. Behaviour manipulation networks established within government were waiting to impose the coming 'Covid' rules and regulations specifically designed to subdue and rewire the psyche of the people in the guise of protecting health. These included in the UK the Behavioural Insights Team part-owned by the British government Cabinet Office; the Scientific Pandemic Insights Group on Behaviours (SPI-B); and a whole web of intelligence and military groups seeking to direct the conversation on social media and control the narrative. Among them are the cyberwarfare (on the people) 77th Brigade of the British military which is also coordinated through the Cabinet Office as civilian and military leadership continues to combine in what they call the Fusion Doctrine. The 77th Brigade is a British equivalent of the infamous Israeli (Sabbatian) military cyberwarfare and Internet manipulation operation Unit 8200 which I expose at length in *The Trigger*. Also carefully in place were the medical and science advisers to government – many on the payroll past or present of Bill Gates – and a whole alternative structure of unelected government stood by to take control when elected parliaments were effectively closed down once the 'Covid' card was slammed on the table. The structure I have described here and so much more was installed in every major country through the Cult networks. The top-down control hierarchy looks like this: The Cult – Cult-owned Gates – the World Health Organization and Tedros – Gates-funded or controlled chief medical officers and science 'advisers' (dictators) in each country –

political 'leaders'– law enforcement – The People. Through this simple global communication and enforcement structure the policy of the Cult could be imposed on virtually the entire human population so long as they acquiesced to the fascism. With everything in place it was time for the button to be pressed in late 2019/early 2020.

These were the prime goals the Cult had to secure for its will to prevail:

1) Locking down economies, closing all but designated 'essential' businesses (Cult-owned corporations were 'essential'), and putting the population under house arrest was an imperative to destroy independent income and employment and ensure dependency on the Cult-controlled state in the Hunger Games Society. Lockdowns had to be established as the global blueprint from the start to respond to the 'virus' and followed by pretty much the entire world.

2) The global population had to be terrified into believing in a deadly 'virus' that didn't actually exist so they would unquestioningly obey authority in the belief that authority must know how best to protect them and their families. Software salesman Gates would suddenly morph into the world's health expert and be promoted as such by the Cult-owned media.

3) A method of testing that wasn't testing for the 'virus', but was only claimed to be, had to be in place to provide the illusion of 'cases' and subsequent 'deaths' that had a very different cause to the 'Covid-19' that would be scribbled on the death certificate.

4) Because there was no 'virus' and the great majority testing positive with a test not testing for the 'virus' would have no symptoms of anything the lie had to be sold that people without symptoms (without the 'virus') could still pass it on to others. This was crucial to justify for the first time quarantining – house arresting – healthy people. Without this the economy-destroying lockdown of *everybody* could not have been credibly sold.

5) The 'saviour' had to be seen as a vaccine which beyond evil drug companies were working like angels of mercy to develop as quickly as possible, with all corners cut, to save the day. The public must absolutely not know that the 'vaccine' had nothing to do with a 'virus' or that the contents were ready and waiting with a very different motive long before the 'Covid' card was even lifted from the pack.

I said in March, 2020, that the 'vaccine' would have been created way ahead of the 'Covid' hoax which justified its use and the following December an article in the New York *Intelligencer* magazine said the Moderna 'vaccine' had been 'designed' by

January, 2020. This was 'before China had even acknowledged that the disease could be transmitted from human to human, more than a week before the first confirmed coronavirus case in the United States'. The article said that by the time the first American death was announced a month later 'the vaccine had already been manufactured and shipped to the National Institutes of Health for the beginning of its Phase I clinical trial'. The 'vaccine' was actually 'designed' long before that although even with this timescale you would expect the article to ask how on earth it could have been done that quickly. Instead it asked why the 'vaccine' had not been rolled out then and not months later. Journalism in the mainstream is truly dead. I am going to detail in the next chapter why the 'virus' has never existed and how a hoax on that scale was possible, but first the foundation on which the Big Lie of 'Covid' was built.

The test that doesn't test

Fraudulent 'testing' is the bottom line of the whole 'Covid' hoax and was the means by which a 'virus' that did not exist *appeared* to exist. They could only achieve this magic trick by using a test not testing for the 'virus'. To use a test that *was* testing for the 'virus' would mean that every test would come back negative given there was no 'virus'. They chose to exploit something called the RT-PCR test invented by American biochemist Kary Mullis in the 1980s who said publicly that his PCR test … *cannot detect infectious disease*. Yes, the 'test' used worldwide to detect infectious 'Covid' to produce all the illusory 'cases' and 'deaths' compiled by Johns Hopkins and others *cannot detect infectious disease*. This fact came from the mouth of the man who invented PCR and was awarded the Nobel Prize in Chemistry in 1993 for doing so. Sadly, and incredibly conveniently for the Cult, Mullis died in August, 2019, at the age of 74 just before his test would be fraudulently used to unleash fascism on the world. He was said to have died from pneumonia which was an irony in itself. A few months later he would have had 'Covid-19' on his death certificate. I say the timing of his death was convenient because had he lived Mullis, a brilliant, honest and decent man, would have been

vociferously speaking out against the use of his test to detect 'Covid' when it was never designed, or able, to do that. I know that to be true given that Mullis made the same point when his test was used to 'detect' – not detect – HIV. He had been seriously critical of the Gallo/Montagnier claim to have isolated the HIV 'virus' and shown it to cause AIDS for which Mullis said there was no evidence. AIDS is actually not a disease but a series of diseases from which people die all the time. When they die from those *same diseases* after a positive 'test' for HIV then AIDS goes on their death certificate. I think I've heard that before somewhere. Countries instigated a policy with 'Covid' that anyone who tested positive with a test not testing for the 'virus' and died of any other cause within 28 days and even longer 'Covid-19' had to go on the death certificate. Cases have come from the test that can't test for infectious disease and the deaths are those who have died of *anything* after testing positive with a test not testing for the 'virus'. I'll have much more later about the death certificate scandal.

Mullis was deeply dismissive of the now US 'Covid' star Anthony Fauci who he said was a liar who didn't know anything about anything – 'and I would say that to his face – nothing.' He said of Fauci: 'The man thinks he can take a blood sample, put it in an electron microscope and if it's got a virus in there you'll know it – he doesn't understand electron microscopy and he doesn't understand medicine and shouldn't be in a position like he's in.' That position, terrifyingly, has made him the decider of 'Covid' fascism policy on behalf of the Cult in his role as director since 1984 of the National Institute of Allergy and Infectious Diseases (NIAID) while his record of being wrong is laughable; but being wrong, so long as it's the *right kind* of wrong, is why the Cult loves him. He'll say anything the Cult tells him to say. Fauci was made Chief Medical Adviser to the President immediately Biden took office. Biden was installed in the White House by Cult manipulation and one of his first decisions was to elevate Fauci to a position of even more control. This is a coincidence? Yes, and I identify as a flamenco dancer called Lola. How does such an incompetent criminal like Fauci remain in that

pivotal position in American health since *the 1980s*? When you serve the Cult it looks after you until you are surplus to requirements. Kary Mullis said prophetically of Fauci and his like: 'Those guys have an agenda and it's not an agenda we would like them to have … they make their own rules, they change them when they want to, and Tony Fauci does not mind going on television in front of the people who pay his salary and lie directly into the camera.' Fauci has done that almost daily since the 'Covid' hoax began. Lying is in Fauci's DNA. To make the situation crystal clear about the PCR test this is a direct quote from its inventor Kary Mullis:

> It [the PCR test] doesn't tell you that you're sick and doesn't tell you that the thing you ended up with was really going to hurt you ...'

Ask yourself why governments and medical systems the world over have been using this very test to decide who is 'infected' with the SARS-CoV-2 'virus' and the alleged disease it allegedly causes, 'Covid-19'. The answer to that question will tell you what has been going on. By the way, here's a little show-stopper – the 'new' SARS-CoV-2 'virus' was 'identified' as such right from the start using … *the PCR test not testing for the 'virus'*. If you are new to this and find that shocking then stick around. I have hardly started yet. Even worse, other 'tests', like the 'Lateral Flow Device' (LFD), are considered so useless that they have to be *confirmed* by the PCR test! Leaked emails written by Ben Dyson, adviser to UK 'Health' Secretary Matt Hancock, said they were 'dangerously unreliable'. Dyson, executive director of strategy at the Department of Health, wrote: 'As of today, someone who gets a positive LFD result in (say) London has at best a 25 per cent chance of it being a true positive, but if it is a self-reported test potentially as low as 10 per cent (on an optimistic assumption about specificity) or as low as 2 per cent (on a more pessimistic assumption).' These are the 'tests' that schoolchildren and the public are being urged to have twice a week or more and have to isolate if they get a positive. Each fake positive goes in the statistics as a 'case' no matter how ludicrously inaccurate and the

'cases' drive lockdown, masks and the pressure to 'vaccinate'. The government said in response to the email leak that the 'tests' were accurate which confirmed yet again what shocking bloody liars they are. The real false positive rate is *100 percent* as we'll see. In another 'you couldn't make it up' the UK government agreed to pay £2.8 billion to California's Innova Medical Group to supply the irrelevant lateral flow tests. The company's primary test-making centre is in China. Innova Medical Group, established in March, 2020, is owned by Pasaca Capital Inc, chaired by Chinese-American millionaire Charles Huang who was born in Wuhan.

How it works – and how it doesn't

The RT-PCR test, known by its full title of Polymerase chain reaction, is used across the world to make millions, even billions, of copies of a DNA/RNA genetic information sample. The process is called 'amplification' and means that a tiny sample of genetic material is amplified to bring out the detailed content. I stress that it is not testing for an infectious disease. It is simply amplifying a sample of genetic material. In the words of Kary Mullis: 'PCR is … just a process that's used to make a whole lot of something out of something.' To emphasise the point companies that make the PCR tests circulated around the world to 'test' for 'Covid' warn on the box that it can't be used to detect 'Covid' or infectious disease and is for research purposes only. It's okay, rest for a minute and you'll be fine. This is the test that produces the 'cases' and 'deaths' that have been used to destroy human society. All those global and national medical and scientific 'experts' demanding this destruction to 'save us' *KNOW* that the test is not testing for the 'virus' and the cases and deaths they claim to be real are an almost unimaginable fraud. Every one of them and so many others including politicians and psychopaths like Gates and Tedros must be brought before Nuremburg-type trials and jailed for the rest of their lives. The more the genetic sample is amplified by PCR the more elements of that material become sensitive to the test and by that I don't mean sensitive for a 'virus' but for elements of the genetic material which

is *naturally* in the body or relates to remnants of old conditions of various kinds lying dormant and causing no disease. Once the amplification of the PCR reaches a certain level *everyone* will test positive. So much of the material has been made sensitive to the test that everyone will have some part of it in their body. Even lying criminals like Fauci have said that once PCR amplifications pass 35 cycles everything will be a false positive that cannot be trusted for the reasons I have described. I say, like many proper doctors and scientists, that 100 percent of the 'positives' are false, but let's just go with Fauci for a moment.

He says that any amplification over 35 cycles will produce false positives and yet the US Centers for Disease Control (CDC) and Food and Drug Administration (FDA) have recommended up to *40 cycles* and the National Health Service (NHS) in Britain admitted in an internal document for staff that it was using *45 cycles* of amplification. A long list of other countries has been doing the same and at least one 'testing' laboratory has been using *50 cycles*. Have you ever heard a doctor, medical 'expert' or the media ask what level of amplification has been used to claim a 'positive'. The 'test' comes back 'positive' and so you have the 'virus', end of story. Now we can see how the government in Tanzania could send off samples from a goat and a pawpaw fruit under human names and both came back positive for 'Covid-19'. Tanzania president John Magufuli mocked the 'Covid' hysteria, the PCR test and masks and refused to import the DNA-manipulating 'vaccine'. The Cult hated him and an article sponsored by the Bill Gates Foundation appeared in the London *Guardian* in February, 2021, headed 'It's time for Africa to rein in Tanzania's anti-vaxxer president'. Well, 'reined in' he shortly was. Magufuli appeared in good health, but then, in March, 2021, he was dead at 61 from 'heart failure'. He was replaced by Samia Hassan Suhulu who is connected to Klaus Schwab's World Economic Forum and she immediately reversed Magufuli's 'Covid' policy. A sample of cola tested positive for 'Covid' with the PCR test in Germany while American actress and singer-songwriter Erykah Badu tested positive in one nostril and negative in the other. Footballer Ronaldo called

the PCR test 'bullshit' after testing positive three times and being forced to quarantine and miss matches when there was nothing wrong with him. The mantra from Tedros at the World Health Organization and national governments (same thing) has been test, test, test. They know that the more tests they can generate the more fake 'cases' they have which go on to become 'deaths' in ways I am coming to. The UK government has its Operation Moonshot planned to test multiple millions every day in workplaces and schools with free tests for everyone to use twice a week at home in line with the Cult plan from the start to make testing part of life. A government advertisement for an 'Interim Head of Asymptomatic Testing Communication' said the job included responsibility for delivering a 'communications strategy' (propaganda) 'to support the expansion of asymptomatic testing that *'normalises testing as part of everyday life*'. More tests means more fake 'cases', 'deaths' and fascism. I have heard of, and from, many people who booked a test, couldn't turn up, and yet got a positive result through the post for a test they'd never even had. The whole thing is crazy, but for the Cult there's method in the madness. Controlling and manipulating the level of amplification of the test means the authorities can control whenever they want the number of apparent 'cases' and 'deaths'. If they want to justify more fascist lockdown and destruction of livelihoods they keep the amplification high. If they want to give the illusion that lockdowns and the 'vaccine' are working then they lower the amplification and 'cases' and 'deaths' will appear to fall. In January, 2021, the Cult-owned World Health Organization suddenly warned laboratories about over-amplification of the test and to lower the threshold. Suddenly headlines began appearing such as: 'Why ARE "Covid" cases plummeting?' This was just when the vaccine rollout was underway and I had predicted months before they would make cases appear to fall through amplification tampering when the 'vaccine' came. These people are so predictable.

Cow vaccines?

The question must be asked of what is on the test swabs being poked far up the nose of the population to the base of the brain? A nasal swab punctured one woman's brain and caused it to leak fluid. Most of these procedures are being done by people with little training or medical knowledge. Dr Lorraine Day, former orthopaedic trauma surgeon and Chief of Orthopaedic Surgery at San Francisco General Hospital, says the tests are really a *'vaccine'*. Cows have long been vaccinated this way. She points out that masks have to cover the nose and the mouth where it is claimed the 'virus' exists in saliva. Why then don't they take saliva from the mouth as they do with a DNA test instead of pushing a long swab up the nose towards the brain? The ethmoid bone separates the nasal cavity from the brain and within that bone is the cribriform plate. Dr Day says that when the swab is pushed up against this plate and twisted the procedure is 'depositing things back there'. She claims that among these 'things' are nanoparticles that can enter the brain. Researchers have noted that a team at the Gates-funded Johns Hopkins have designed tiny, star-shaped micro-devices that can latch onto intestinal mucosa and release drugs into the body. Mucosa is the thin skin that covers the inside surface of parts of the body such as *the nose* and mouth and produces mucus to protect them. The Johns Hopkins micro-devices are called 'theragrippers' and were 'inspired' by a parasitic worm that digs its sharp teeth into a host's intestines. Nasal swabs are also coated in the sterilisation agent ethylene oxide. The US National Cancer Institute posts this explanation on its website:

> At room temperature, ethylene oxide is a flammable colorless gas with a sweet odor. It is used primarily to produce other chemicals, including antifreeze. In smaller amounts, ethylene oxide is used as a pesticide and a sterilizing agent. The ability of ethylene oxide to damage DNA makes it an effective sterilizing agent but also accounts for its cancer-causing activity.

The Institute mentions lymphoma and leukaemia as cancers most frequently reported to be associated with occupational exposure to ethylene oxide along with stomach and breast cancers. How does anyone think this is going to work out with the constant testing

regime being inflicted on adults and children at home and at school that will accumulate in the body anything that's on the swab?

Doctors know best

It is vital for people to realise that 'hero' doctors 'know' only what the Big Pharma-dominated medical authorities tell them to 'know' and if they refuse to 'know' what they are told to 'know' they are out the door. They are mostly not physicians or healers, but repeaters of the official narrative – or else. I have seen alleged professional doctors on British television make shocking statements that we are supposed to take seriously. One called 'Dr' Amir Khan, who is actually telling patients how to respond to illness, said that men could take the birth pill to 'help slow down the effects of Covid-19'. In March, 2021, another ridiculous 'Covid study' by an American doctor proposed injecting men with the female sex hormone progesterone as a 'Covid' treatment. British doctor Nighat Arif told the BBC that face coverings were now going to be part of ongoing normal. Yes, the vaccine protects you, she said (evidence?) … but the way to deal with viruses in the community was always going to come down to hand washing, face covering and keeping a physical distance. That's not what we were told before the 'vaccine' was circulating. Arif said she couldn't imagine ever again going on the underground or in a lift without a mask. I was just thanking my good luck that she was not my doctor when she said – in March, 2021 – that if 'we are *behaving* and we are doing all the right things' she thought we could 'have our nearest and dearest around us at home … around *Christmas* and N*ew Year!* Her patronising delivery was the usual school teacher talking to six-year-olds as she repeated every government talking point and probably believed them all. If we have learned anything from the 'Covid' experience surely it must be that humanity's perception of doctors needs a fundamental rethink. NHS 'doctor' Sara Kayat told her television audience that the 'Covid vaccine' would '100 percent prevent hospitalisation and death'. Not even Big Pharma claimed that. We have to stop taking 'experts' at their word without question when so many of them are

clueless and only repeating the party line on which their careers depend. That is not to say there are not brilliants doctors – there are and I have spoken to many of them since all this began – but you won't see them in the mainstream media or quoted by the psychopaths and yes-people in government.

Remember the name – Christian Drosten

German virologist Christian Drosten, Director of Charité Institute of Virology in Berlin, became a national star after the pandemic hoax began. He was feted on television and advised the German government on 'Covid' policy. Most importantly to the wider world Drosten led a group that produced the 'Covid' testing protocol for the PCR test. What a remarkable feat given the PCR cannot test for infectious disease and even more so when you think that Drosten said that his method of testing for SARS-CoV-2 was developed 'without having virus material available'. *He developed a test for a 'virus' that he didn't have and had never seen*. Let that sink in as you survey the global devastation that came from what he did. The whole catastrophe of Drosten's 'test' was based on the alleged genetic sequence published by Chinese scientists on the Internet. We will see in the next chapter that this alleged 'genetic sequence' has never been produced by China or anyone and cannot be when there *is no* SARS-CoV-2. Drosten, however, doesn't seem to let little details like that get in the way. He was the lead author with Victor Corman from the same Charité Hospital of the paper 'Detection of 2019 novel coronavirus (2019-nCoV) by real-time PCR' published in a magazine called *Eurosurveillance*. This became known as the Corman-Drosten paper. In November, 2020, with human society devastated by the effects of the Corman-Drosten test baloney, the protocol was publicly challenged by 22 international scientists and independent researchers from Europe, the United States, and Japan. Among them were senior molecular geneticists, biochemists, immunologists, and microbiologists. They produced a document headed 'External peer review of the RTPCR test to detect SARS-Cov-2 Reveals 10 Major Flaws At The Molecular and Methodological Level: Consequences

For False-Positive Results'. The flaws in the Corman-Drosten test included the following:

- The test is non-specific because of erroneous design
- Results are enormously variable
- The test is unable to discriminate between the whole 'virus' and viral fragments
- It doesn't have positive or negative controls
- The test lacks a standard operating procedure
- It is unsupported by proper peer view

The scientists said the PCR 'Covid' testing protocol was not founded on science and they demanded the Corman-Drosten paper be retracted by *Eurosurveillance*. They said all present and previous Covid deaths, cases, and 'infection rates' should be subject to a massive retroactive inquiry. Lockdowns and travel restrictions should be reviewed and relaxed and those diagnosed through PCR to have 'Covid-19' should not be forced to isolate. Dr Kevin Corbett, a health researcher and nurse educator with a long academic career producing a stream of peer-reviewed publications at many UK universities, made the same point about the PCR test debacle. He said of the scientists' conclusions: 'Every scientific rationale for the development of that test has been totally destroyed by this paper. It's like Hiroshima/Nagasaki to the Covid test.' He said that China hadn't given them an isolated 'virus' when Drosten developed the test. Instead they had developed the test from *a sequence in a gene bank*.' Put another way … *they made it up*! The scientists were supported in this contention by a Portuguese appeals court which ruled in November, 2020, that PCR tests are unreliable and it is unlawful to quarantine people based solely on a PCR test. The point about China not providing an isolated virus must be true when the 'virus' has never been isolated to this day and the consequences of that will become clear. Drosten and company produced this useless 'protocol' right on cue in January, 2020, just as the 'virus' was said to

be moving westward and it somehow managed to successfully pass a peer-review in 24 hours. In other words there was no peer-review for a test that would be used to decide who had 'Covid' and who didn't across the world. The Cult-created, Gates-controlled World Health Organization immediately recommended all its nearly 200 member countries to use the Drosten PCR protocol to detect 'cases' and 'deaths'. The sting was underway and it continues to this day.

So who is this Christian Drosten that produced the means through which death, destruction and economic catastrophe would be justified? His education background, including his doctoral thesis, would appear to be somewhat shrouded in mystery and his track record is dire as with another essential player in the 'Covid' hoax, the Gates-funded Professor Neil Ferguson at the Gates-funded Imperial College in London of whom more shortly. Drosten predicted in 2003 that the alleged original SARS 'virus' (SARS-1') was an epidemic that could have serious effects on economies and an effective vaccine would take at least two years to produce. Drosten's answer to every alleged 'outbreak' is a vaccine which you won't be shocked to know. What followed were just 774 official deaths worldwide and none in Germany where there were only nine cases. That is even if you believe there ever was a SARS 'virus' when the evidence is zilch and I will expand on this in the next chapter. Drosten claims to be co-discoverer of 'SARS-1' and developed a test for it in 2003. He was screaming warnings about 'swine flu' in 2009 and how it was a widespread infection far more severe than any dangers from a vaccine could be and people should get vaccinated. It would be helpful for Drosten's vocal chords if he simply recorded the words 'the virus is deadly and you need to get vaccinated' and copies could be handed out whenever the latest made-up threat comes along. Drosten's swine flu epidemic never happened, but Big Pharma didn't mind with governments spending hundreds of millions on vaccines that hardly anyone bothered to use and many who did wished they hadn't. A study in 2010 revealed that the risk of dying from swine flu, or H1N1, was no higher than that of the annual seasonal flu which is what at least most of 'it' really was as in

the case of 'Covid-19'. A media investigation into Drosten asked how with such a record of inaccuracy he could be *the* government adviser on these issues. The answer to that question is the same with Drosten, Ferguson and Fauci – they keep on giving the authorities the 'conclusions' and 'advice' they want to hear. Drosten certainly produced the goods for them in January, 2020, with his PCR protocol garbage and provided the foundation of what German internal medicine specialist Dr Claus Köhnlein, co-author of *Virus Mania*, called the 'test pandemic'. The 22 scientists in the *Eurosurveillance* challenge called out conflicts of interest within the Drosten 'protocol' group and with good reason. Olfert Landt, a regular co-author of Drosten 'studies', owns the biotech company TIB Molbiol Syntheselabor GmbH in Berlin which manufactures and sells the tests that Drosten and his mates come up with. They have done this with SARS, Enterotoxigenic E. coli (ETEC), MERS, Zika 'virus', yellow fever, and now 'Covid'. Landt told the *Berliner Zeitung* newspaper:

> The testing, design and development came from the Charité [Drosten and Corman]. We simply implemented it immediately in the form of a kit. And if we don't have the virus, which originally only existed in Wuhan, we can make a synthetic gene to simulate the genome of the virus. That's what we did very quickly.

This is more confirmation that the Drosten test was designed without access to the 'virus' and only a synthetic simulation which is what SARS-CoV-2 really is – a computer-generated synthetic fiction. It's quite an enterprise they have going here. A Drosten team decides what the test for something should be and Landt's biotech company flogs it to governments and medical systems across the world. His company must have made an absolute fortune since the 'Covid' hoax began. Dr Reiner Fuellmich, a prominent German consumer protection trial lawyer in Germany and California, is on Drosten's case and that of Tedros at the World Health Organization for crimes against humanity with a class-action lawsuit being prepared in the United States and other legal action in Germany.

Why China?

Scamming the world with a 'virus' that doesn't exist would seem impossible on the face of it, but not if you have control of the relatively few people that make policy decisions and the great majority of the global media. Remember it's not about changing 'real' reality it's about controlling *perception* of reality. You don't have to make something happen you only have make people *believe* that it's happening. Renegade Minds understand this and are therefore much harder to swindle. 'Covid-19' is not a 'real' 'virus'. It's a mind virus, like a computer virus, which has infected the minds, not the bodies, of billions. It all started, publically at least, in China and that alone is of central significance. The Cult was behind the revolution led by its asset Mao Zedong, or Chairman Mao, which established the People's Republic of China on October 1st, 1949. It should have been called The Cult's Republic of China, but the name had to reflect the recurring illusion that vicious dictatorships are run by and for the people (see all the 'Democratic Republics' controlled by tyrants). In the same way we have the 'Biden' Democratic Republic of America officially ruled by a puppet tyrant (at least temporarily) on behalf of Cult tyrants. The creation of Mao's merciless communist/fascist dictatorship was part of a frenzy of activity by the Cult at the conclusion of World War Two which, like the First World War, it had instigated through its assets in Germany, Britain, France, the United States and elsewhere. Israel was formed in 1948; the Soviet Union expanded its 'Iron Curtain' control, influence and military power with the Warsaw Pact communist alliance in 1955; the United Nations was formed in 1945 as a Cult precursor to world government; and a long list of world bodies would be established including the World Health Organization (1948), World Trade Organization (1948 under another name until 1995), International Monetary Fund (1945) and World Bank (1944). Human society was redrawn and hugely centralised in the global Problem-Reaction-Solution that was World War Two. All these changes were significant. Israel would become the headquarters of the Sabbatians

and the revolution in China would prepare the ground and control system for the events of 2019/2020.

Renegade Minds know there are no borders except for public consumption. The Cult is a seamless, borderless global entity and to understand the game we need to put aside labels like borders, nations, countries, communism, fascism and democracy. These delude the population into believing that countries are ruled within their borders by a government of whatever shade when these are mere agencies of a global power. America's illusion of democracy and China's communism/fascism are subsidiaries – vehicles – for the same agenda. We may hear about conflict and competition between America and China and on the lower levels that will be true; but at the Cult level they are branches of the same company in the way of the McDonald's example I gave earlier. I have tracked in the books over the years support by US governments of both parties for Chinese Communist Party infiltration of American society through allowing the sale of land, even military facilities, and the acquisition of American business and university influence. All this is underpinned by the infamous stealing of intellectual property and technological know-how. Cult-owned Silicon Valley corporations waive their fraudulent 'morality' to do business with human-rights-free China; Cult-controlled Disney has become China's PR department; and China in effect owns 'American' sports such as basketball which depends for much of its income on Chinese audiences. As a result any sports player, coach or official speaking out against China's horrific human rights record is immediately condemned or fired by the China-worshipping National Basketball Association. One of the first acts of China-controlled Biden was to issue an executive order telling federal agencies to stop making references to the 'virus' by the 'geographic location of its origin'. Long-time Congressman Jerry Nadler warned that criticising China, America's biggest rival, leads to hate crimes against Asian people in the United States. So shut up you bigot. China is fast closing in on Israel as a country that must not be criticised which is apt, really, given that Sabbatians control them both. The two countries have

developed close economic, military, technological and strategic ties which include involvement in China's 'Silk Road' transport and economic initiative to connect China with Europe. Israel was the first country in the Middle East to recognise the establishment of Mao's tyranny in 1950 months after it was established.

Project Wuhan – the 'Covid' Psyop

I emphasise again that the Cult plays the long game and what is happening to the world today is the result of centuries of calculated manipulation following a script to take control step-by-step of every aspect of human society. I will discuss later the common force behind all this that has spanned those centuries and thousands of years if the truth be told. Instigating the Mao revolution in China in 1949 with a 2020 'pandemic' in mind is not only how they work – the 71 years between them is really quite short by the Cult's standards of manipulation preparation. The reason for the Cult's Chinese revolution was to create a fiercely-controlled environment within which an extreme structure for human control could be incubated to eventually be unleashed across the world. We have seen this happen since the 'pandemic' emerged from China with the Chinese control-structure founded on AI technology and tyrannical enforcement sweep across the West. Until the moment when the Cult went for broke in the West and put its fascism on public display Western governments had to pay some lip-service to freedom and democracy to not alert too many people to the tyranny-in-the-making. Freedoms were more subtly eroded and power centralised with covert government structures put in place waiting for the arrival of 2020 when that smokescreen of 'freedom' could be dispensed with. The West was not able to move towards tyranny before 2020 anything like as fast as China which was created as a tyranny and had no limits on how fast it could construct the Cult's blueprint for global control. When the time came to impose that structure on the world it was the same Cult-owned Chinese communist/fascist government that provided the excuse – the 'Covid pandemic'. It was absolutely crucial to the Cult plan for the Chinese response to the 'pandemic' –

draconian lockdowns of the entire population – to become the blueprint that Western countries would follow to destroy the livelihoods and freedom of their people. This is why the Cult-owned, Gates-owned, WHO Director-General Tedros said early on:

The Chinese government is to be congratulated for the extraordinary measures it has taken to contain the outbreak. China is actually setting a new standard for outbreak response and it is not an exaggeration.

Forbes magazine said of China: '… those measures protected untold millions from getting the disease'. The Rockefeller Foundation 'epidemic scenario' document in 2010 said 'prophetically':

However, a few countries did fare better – China in particular. The Chinese government's quick imposition and enforcement of mandatory quarantine for all citizens, as well as its instant and near-hermetic sealing off of all borders, saved millions of lives, stopping the spread of the virus far earlier than in other countries and enabling a swifter post-pandemic recovery.

Once again – *spooky*.

The first official story was the 'bat theory' or rather the bat diversion. The source of the 'virus outbreak' we were told was a ''wet market' in Wuhan where bats and other animals are bought and eaten in horrifically unhygienic conditions. Then another story emerged through the alternative media that the 'virus' had been released on purpose or by accident from a BSL-4 (biosafety level 4) laboratory in Wuhan not far from the wet market. The lab was reported to create and work with lethal concoctions and bioweapons. Biosafety level 4 is the highest in the World Health Organization system of safety and containment. Renegade Minds are aware of what I call designer manipulation. The ideal for the Cult is for people to buy its prime narrative which in the opening salvoes of the 'pandemic' was the wet market story. It knows, however, that there is now a considerable worldwide alternative media of researchers sceptical of anything governments say and they are often given a version of events in a form they can perceive as credible while misdirecting them from the real truth. In this case let them

think that the conspiracy involved is a 'bioweapon virus' released from the Wuhan lab to keep them from the real conspiracy – *there is no 'virus'*. The WHO's current position on the source of the outbreak at the time of writing appears to be: 'We haven't got a clue, mate.' This is a good position to maintain mystery and bewilderment. The inner circle will know where the 'virus' came from – *nowhere*. The bottom line was to ensure the public believed there *was* a 'virus' and it didn't much matter if they thought it was natural or had been released from a lab. The belief that there was a 'deadly virus' was all that was needed to trigger global panic and fear. The population was terrified into handing their power to authority and doing what they were told. They had to or they were 'all gonna die'.

In March, 2020, information began to come my way from real doctors and scientists and my own additional research which had my intuition screaming: 'Yes, that's it! *There is no virus*.' The 'bioweapon' was not the 'virus'; it was the *'vaccine'* already being talked about that would be the bioweapon. My conclusion was further enhanced by happenings in Wuhan. The 'virus' was said to be sweeping the city and news footage circulated of people collapsing in the street (which they've never done in the West with the same 'virus'). The Chinese government was building 'new hospitals' in a matter of ten days to 'cope with demand' such was the virulent nature of the 'virus'. Yet in what seemed like no time the 'new hospitals' closed – even if they even opened – and China declared itself 'virus-free'. It was back to business as usual. This was more propaganda to promote the Chinese draconian lockdowns in the West as the way to 'beat the virus'. Trouble was that we subsequently had lockdown after lockdown, but never business as usual. As the people of the West and most of the rest of the world were caught in an ever-worsening spiral of lockdown, social distancing, masks, isolated old people, families forced apart, and livelihood destruction, it was party-time in Wuhan. Pictures emerged of thousands of people enjoying pool parties and concerts. It made no sense until you realised there never was a 'virus' and the

whole thing was a Cult set-up to transform human society out of one its major global strongholds – China.

How is it possible to deceive virtually the entire world population into believing there is a deadly virus when there is not even a 'virus' let alone a deadly one? It's nothing like as difficult as you would think and that's clearly true because it happened.

Postscript: See end of book Postscript for more on the 'Wuhan lab virus release' story which the authorities and media were pushing heavily in the summer of 2021 to divert attention from the truth that the 'Covid virus' is pure invention.

CHAPTER FIVE

There *is no* 'virus'

You can fool some of the people all of the time, and all of the people some of the time, but you cannot fool all of the people all of the time
Abraham Lincoln

The greatest form of mind control is repetition. The more you repeat the same mantra of alleged 'facts' the more will accept them to be true. It becomes an 'everyone knows that, mate'. If you can also censor any other version or alternative to your alleged 'facts' you are pretty much home and cooking.

By the start of 2020 the Cult owned the global mainstream media almost in its entirety to spew out its 'Covid' propaganda and ignore or discredit any other information and view. Cult-owned social media platforms in Cult-owned Silicon Valley were poised and ready to unleash a campaign of ferocious censorship to obliterate all but the official narrative. To complete the circle many demands for censorship by Silicon Valley were led by the mainstream media as 'journalists' became full-out enforcers for the Cult both as propagandists and censors. Part of this has been the influx of young people straight out of university who have become 'journalists' in significant positions. They have no experience and a headful of programmed perceptions from their years at school and university at a time when today's young are the most perceptually-targeted generations in known human history given the insidious impact of technology. They enter the media perceptually prepared and ready to repeat the narratives of the system that programmed them to

repeat its narratives. The BBC has a truly pathetic 'specialist disinformation reporter' called Marianna Spring who fits this bill perfectly. She is clueless about the world, how it works and what is really going on. Her role is to discredit anyone doing the job that a proper journalist would do and system-serving hacks like Spring wouldn't dare to do or even see the need to do. They are too busy licking the arse of authority which can never be wrong and, in the case of the BBC propaganda programme, *Panorama*, contacting payments systems such as PayPal to have a donations page taken down for a film company making documentaries questioning vaccines. Even the BBC soap opera *EastEnders* included a disgracefully biased scene in which an inarticulate white working class woman was made to look foolish for questioning the 'vaccine' while a well-spoken black man and Asian woman promoted the government narrative. It ticked every BBC box and the fact that the black and minority community was resisting the 'vaccine' had nothing to do with the way the scene was written. The BBC has become a disgusting tyrannical propaganda and censorship operation that should be defunded and disbanded and a free media take its place with a brief to stop censorship instead of demanding it. A BBC 'interview' with Gates goes something like: 'Mr Gates, sir, if I can call you sir, would you like to tell our audience why you are such a great man, a wonderful humanitarian philanthropist, and why you should absolutely be allowed as a software salesman to decide health policy for approaching eight billion people? Thank you, sir, please sir.' Propaganda programming has been incessant and merciless and when all you hear is the same story from the media, repeated by those around you who have only heard the same story, is it any wonder that people on a grand scale believe absolute mendacious garbage to be true? You are about to see, too, why this level of information control is necessary when the official 'Covid' narrative is so nonsensical and unsupportable by the evidence.

Structure of Deceit

The pyramid structure through which the 'Covid' hoax has been manifested is very simple and has to be to work. As few people as possible have to be involved with full knowledge of what they are doing – and why – or the real story would get out. At the top of the pyramid are the inner core of the Cult which controls Bill Gates who, in turn, controls the World Health Organization through his pivotal funding and his puppet Director-General mouthpiece, Tedros. Before he was appointed Tedros was chair of the Gates-founded Global Fund to 'fight against AIDS, tuberculosis and malaria', a board member of the Gates-funded 'vaccine alliance' GAVI, and on the board of another Gates-funded organisation. Gates owns him and picked him for a specific reason – Tedros is a crook and worse. 'Dr' Tedros (he's not a medical doctor, the first WHO chief not to be) was a member of the tyrannical Marxist government of Ethiopia for decades with all its human rights abuses. He has faced allegations of corruption and misappropriation of funds and was exposed three times for covering up cholera epidemics while Ethiopia's health minister. Tedros appointed the mass-murdering genocidal Zimbabwe dictator Robert Mugabe as a WHO goodwill ambassador for public health which, as with Tedros, is like appointing a psychopath to run a peace and love campaign. The move was so ridiculous that he had to drop Mugabe in the face of widespread condemnation. American economist David Steinman, a Nobel peace prize nominee, lodged a complaint with the International Criminal Court in The Hague over alleged genocide by Tedros when he was Ethiopia's foreign minister. Steinman says Tedros was a 'crucial decision maker' who directed the actions of Ethiopia's security forces from 2013 to 2015 and one of three officials in charge when those security services embarked on the 'killing' and 'torturing' of Ethiopians. You can see where Tedros is coming from and it's sobering to think that he has been the vehicle for Gates and the Cult to direct the global response to 'Covid'. Think about that. A psychopathic Cult dictates to psychopath Gates who dictates to psychopath Tedros who dictates how countries of the world must respond to a 'Covid virus' never scientifically shown to exist. At the same time psychopathic Cult-owned Silicon Valley information

giants like Google, YouTube, Facebook and Twitter announced very early on that they would give the Cult/Gates/Tedros/WHO version of the narrative free advertising and censor those who challenged their intelligence-insulting, mendacious story.

The next layer in the global 'medical' structure below the Cult, Gates and Tedros are the chief medical officers and science 'advisers' in each of the WHO member countries which means virtually all of them. Medical officers and arbiters of science (they're not) then take the WHO policy and recommended responses and impose them on their country's population while the political 'leaders' say they are deciding policy (they're clearly not) by 'following the science' on the advice of the 'experts' – the same medical officers and science 'advisers' (dictators). In this way with the rarest of exceptions the entire world followed the same policy of lockdown, people distancing, masks and 'vaccines' dictated by the psychopathic Cult, psychopathic Gates and psychopathic Tedros who we are supposed to believe give a damn about the health of the world population they are seeking to enslave. That, amazingly, is all there is to it in terms of crucial decision-making. Medical staff in each country then follow like sheep the dictates of the shepherds at the top of the national medical hierarchies – chief medical officers and science 'advisers' who themselves follow like sheep the shepherds of the World Health Organization and the Cult. Shepherds at the national level often have major funding and other connections to Gates and his Bill and Melinda Gates Foundation which carefully hands out money like confetti at a wedding to control the entire global medical system from the WHO down.

Follow the money

Christopher Whitty, Chief Medical Adviser to the UK Government at the centre of 'virus' policy, a senior adviser to the government's Scientific Advisory Group for Emergencies (SAGE), and Executive Board member of the World Health Organization, was gifted a grant of $40 million by the Bill and Melinda Gates Foundation for malaria research in Africa. The BBC described the unelected Whitty as 'the

official who will probably have the greatest impact on our everyday lives of any individual policymaker in modern times' and so it turned out. What Gates and Tedros have said Whitty has done like his equivalents around the world. Patrick Vallance, co-chair of SAGE and the government's Chief Scientific Adviser, is a former executive of Big Pharma giant GlaxoSmithKline with its fundamental financial and business connections to Bill Gates. In September, 2020, it was revealed that Vallance owned a deferred bonus of shares in GlaxoSmithKline worth £600,000 while the company was 'developing' a 'Covid vaccine'. Move along now – nothing to see here – what could possibly be wrong with that? Imperial College in London, a major player in 'Covid' policy in Britain and elsewhere with its 'Covid-19' Response Team, is funded by Gates and has big connections to China while the now infamous Professor Neil Ferguson, the useless 'computer modeller' at Imperial College is also funded by Gates. Ferguson delivered the dramatically inaccurate excuse for the first lockdowns (much more in the next chapter). The Institute for Health Metrics and Evaluation (IHME) in the United States, another source of outrageously false 'Covid' computer models to justify lockdowns, is bankrolled by Gates who is a vehement promotor of lockdowns. America's version of Whitty and Vallance, the again now infamous Anthony Fauci, has connections to 'Covid vaccine' maker Moderna as does Bill Gates through funding from the Bill and Melinda Gates Foundation. Fauci is director of the National Institute of Allergy and Infectious Diseases (NIAID), a major recipient of Gates money, and they are very close. Deborah Birx who was appointed White House Coronavirus Response Coordinator in February, 2020, is yet another with ties to Gates. Everywhere you look at the different elements around the world behind the coordination and decision making of the 'Covid' hoax there is Bill Gates and his money. They include the World Health Organization; Centers for Disease Control (CDC) in the United States; National Institutes of Health (NIH) of Anthony Fauci; Imperial College and Neil Ferguson; the London School of Hygiene where Chris Whitty worked; Regulatory agencies like the UK Medicines & Healthcare products Regulatory Agency (MHRA)

which gave emergency approval for 'Covid vaccines'; Wellcome Trust; GAVI, the Vaccine Alliance; the Coalition for Epidemic Preparedness Innovations (CEPI); Johns Hopkins University which has compiled the false 'Covid' figures; and the World Economic Forum. A Nationalfile.com article said:

> Gates has a lot of pull in the medical world, he has a multi-million dollar relationship with Dr. Fauci, and Fauci originally took the Gates line supporting vaccines and casting doubt on [the drug hydroxychloroquine]. Coronavirus response team member Dr. Deborah Birx, appointed by former president Obama to serve as United States Global AIDS Coordinator, also sits on the board of a group that has received billions from Gates' foundation, and Birx reportedly used a disputed Bill Gates-funded model for the White House's Coronavirus effort. Gates is a big proponent for a population lockdown scenario for the Coronavirus outbreak.

Another funder of Moderna is the Defense Advanced Research Projects Agency (DARPA), the technology-development arm of the Pentagon and one of the most sinister organisations on earth. DARPA had a major role with the CIA covert technology-funding operation In-Q-Tel in the development of Google and social media which is now at the centre of global censorship. Fauci and Gates are extremely close and openly admit to talking regularly about 'Covid' policy, but then why wouldn't Gates have a seat at every national 'Covid' table after his Foundation committed $1.75 billion to the 'fight against Covid-19'. When passed through our Orwellian Translation Unit this means that he has bought and paid for the Cult-driven 'Covid' response worldwide. Research the major 'Covid' response personnel in your own country and you will find the same Gates funding and other connections again and again. Medical and science chiefs following World Health Organization 'policy' sit atop a medical hierarchy in their country of administrators, doctors and nursing staff. These 'subordinates' are told they must work and behave in accordance with the policy delivered from the 'top' of the national 'health' pyramid which is largely the policy delivered by the WHO which is the policy delivered by Gates and the Cult. The whole 'Covid' narrative has been imposed on medical staff by a climate of fear although great numbers don't even need that to comply. They do so through breathtaking levels of ignorance and

include doctors who go through life simply repeating what Big Pharma and their hierarchical masters tell them to say and believe. No wonder Big Pharma 'medicine' is one of the biggest killers on Planet Earth.

The same top-down system of intimidation operates with regard to the Cult Big Pharma cartel which also dictates policy through national and global medical systems in this way. The Cult and Big Pharma agendas are the same because the former controls and owns the latter. 'Health' administrators, doctors, and nursing staff are told to support and parrot the dictated policy or they will face consequences which can include being fired. How sad it's been to see medical staff meekly repeating and imposing Cult policy without question and most of those who can see through the deceit are only willing to speak anonymously off the record. They know what will happen if their identity is known. This has left the courageous few to expose the lies about the 'virus', face masks, overwhelmed hospitals that aren't, and the dangers of the 'vaccine' that isn't a vaccine. When these medical professionals and scientists, some renowned in their field, have taken to the Internet to expose the truth their articles, comments and videos have been deleted by Cult-owned Facebook, Twitter and YouTube. What a real head-shaker to see YouTube videos with leading world scientists and highly qualified medical specialists with an added link underneath to the notorious Cult propaganda website *Wikipedia* to find the 'facts' about the same subject.

HIV – the 'Covid' trial-run

I'll give you an example of the consequences for health and truth that come from censorship and unquestioning belief in official narratives. The story was told by PCR inventor Kary Mullis in his book *Dancing Naked in the Mind Field*. He said that in 1984 he accepted as just another scientific fact that Luc Montagnier of France's Pasteur Institute and Robert Gallo of America's National Institutes of Health had independently discovered that a 'retrovirus' dubbed HIV (human immunodeficiency virus) caused AIDS. They

were, after all, Mullis writes, specialists in retroviruses. This is how the medical and science pyramids work. Something is announced or *assumed* and then becomes an everybody-knows-that purely through repetition of the assumption as if it is fact. Complete crap becomes accepted truth with no supporting evidence and only repetition of the crap. This is how a 'virus' that doesn't exist became the 'virus' that changed the world. The HIV-AIDS fairy story became a multi-billion pound industry and the media poured out propaganda terrifying the world about the deadly HIV 'virus' that caused the lethal AIDS. By then Mullis was working at a lab in Santa Monica, California, to detect retroviruses with his PCR test in blood donations received by the Red Cross. In doing so he asked a virologist where he could find a reference for HIV being the cause of AIDS. 'You don't need a reference,' the virologist said … *'Everybody knows it.'* Mullis said he wanted to quote a reference in the report he was doing and he said he felt a little funny about not knowing the source of such an important discovery when everyone else seemed to. The virologist suggested he cite a report by the Centers for Disease Control and Prevention (CDC) on morbidity and mortality. Mullis read the report, but it only said that an organism had been identified and did not say how. The report did not identify the original scientific work. Physicians, however, *assumed* (key recurring theme) that if the CDC was convinced that HIV caused AIDS then proof must exist. Mullis continues:

I did computer searches. Neither Montagnier, Gallo, nor anyone else had published papers describing experiments which led to the conclusion that HIV probably caused AIDS. I read the papers in Science for which they had become well known as AIDS doctors, but all they had said there was that they had found evidence of a past infection by something which was probably HIV in some AIDS patients.

They found antibodies. Antibodies to viruses had always been considered evidence of past disease, not present disease. Antibodies signaled that the virus had been defeated. The patient had saved himself. There was no indication in these papers that this virus caused a disease. They didn't show that everybody with the antibodies had the disease. In fact they found some healthy people with antibodies.

Mullis asked why their work had been published if Montagnier and Gallo hadn't really found this evidence, and why had they been fighting so hard to get credit for the discovery? He says he was hesitant to write 'HIV is the probable cause of AIDS' until he found published evidence to support that. 'Tens of thousands of scientists and researchers were spending billions of dollars a year doing research based on this idea,' Mullis writes. 'The reason had to be there somewhere; otherwise these people would not have allowed their research to settle into one narrow channel of investigation.' He said he lectured about PCR at numerous meetings where people were always talking about HIV and he asked them how they knew that HIV was the cause of AIDS:

> Everyone said something. Everyone had the answer at home, in the office, in some drawer. They all knew, and they would send me the papers as soon as they got back. But I never got any papers. Nobody ever sent me the news about how AIDS was caused by HIV.

Eventually Mullis was able to ask Montagnier himself about the reference proof when he lectured in San Diego at the grand opening of the University of California AIDS Research Center. Mullis says this was the last time he would ask his question without showing anger. Montagnier said he should reference the CDC report. 'I read it', Mullis said, and it didn't answer the question. 'If Montagnier didn't know the answer who the hell did?' Then one night Mullis was driving when an interview came on National Public Radio with Peter Duesberg, a prominent virologist at Berkeley and a California Scientist of the Year. Mullis says he finally understood why he could not find references that connected HIV to AIDS – *there weren't any!* No one had ever proved that HIV causes AIDS even though it had spawned a multi-billion pound global industry and the media was repeating this as fact every day in their articles and broadcasts terrifying the shit out of people about AIDS and giving the impression that a positive test for HIV (see 'Covid') was a death sentence. Duesberg was a threat to the AIDS gravy train and the agenda that underpinned it. He was therefore abused and castigated after he told the Proceedings of the National Academy of Sciences

there was no good evidence implicating the new 'virus'. Editors rejected his manuscripts and his research funds were deleted. Mullis points out that the CDC has defined AIDS as one of more than 30 diseases *if accompanied* by a positive result on a test that detects antibodies to HIV; but those same diseases are not defined as AIDS cases when antibodies are not detected:

> If an HIV-positive woman develops uterine cancer, for example, she is considered to have AIDS. If she is not HIV positive, she simply has uterine cancer. An HIV-positive man with tuberculosis has AIDS; if he tests negative he simply has tuberculosis. If he lives in Kenya or Colombia, where the test for HIV antibodies is too expensive, he is simply presumed to have the antibodies and therefore AIDS, and therefore he can be treated in the World Health Organization's clinic. It's the only medical help available in some places. And it's free, because the countries that support WHO are worried about AIDS.

Mullis accuses the CDC of continually adding new diseases (see ever more 'Covid symptoms') to the grand AIDS definition and of virtually doctoring the books to make it appear as if the disease continued to spread. He cites how in 1993 the CDC enormously broadened its AIDS definition and county health authorities were delighted because they received $2,500 per year from the Federal government for every reported AIDS case. Ladies and gentlemen, I have just described, via Kary Mullis, the 'Covid pandemic' of 2020 and beyond. Every element is the same and it's been pulled off in the same way by the same networks.

The 'Covid virus' exists? Okay – prove it. Er … still waiting

What Kary Mullis described with regard to 'HIV' has been repeated with 'Covid'. A claim is made that a new, or 'novel', infection has been found and the entire medical system of the world repeats that as fact exactly as they did with HIV and AIDS. No one in the mainstream asks rather relevant questions such as 'How do you know?' and 'Where is your proof?' The SARS-Cov-2 'virus' and the 'Covid-19 disease' became an overnight 'everybody-knows-that'. The origin could be debated and mulled over, but what you could not suggest was that 'SARS-Cov-2' didn't exist. That would be

ridiculous. 'Everybody knows' the 'virus' exists. Well, I didn't for one along with American proper doctors like Andrew Kaufman and Tom Cowan and long-time American proper journalist Jon Rappaport. We dared to pursue the obvious and simple question: 'Where's the evidence?' The overwhelming majority in medicine, journalism and the general public did not think to ask that. After all, *everyone knew* there was a new 'virus'. Everyone was saying so and I heard it on the BBC. Some would eventually argue that the 'deadly virus' was nothing like as deadly as claimed, but few would venture into the realms of its very existence. Had they done so they would have found that the evidence for that claim had gone AWOL as with HIV causes AIDS. In fact, not even that. For something to go AWOL it has to exist in the first place and scientific proof for a 'SARS-Cov-2' can be filed under nothing, nowhere and zilch.

Dr Andrew Kaufman is a board-certified forensic psychiatrist in New York State, a Doctor of Medicine and former Assistant Professor and Medical Director of Psychiatry at SUNY Upstate Medical University, and Medical Instructor of Hematology and Oncology at the Medical School of South Carolina. He also studied biology at the Massachusetts Institute of Technology (MIT) and trained in Psychiatry at Duke University. Kaufman is retired from allopathic medicine, but remains a consultant and educator on natural healing, I saw a video of his very early on in the 'Covid' hoax in which he questioned claims about the 'virus' in the absence of any supporting evidence and with plenty pointing the other way. I did everything I could to circulate his work which I felt was asking the pivotal questions that needed an answer. I can recommend an excellent pull-together interview he did with the website The Last Vagabond entitled *Dr Andrew Kaufman: Virus Isolation, Terrain Theory and Covid-19* and his website is andrewkaufmanmd.com. Kaufman is not only a forensic psychiatrist; he is forensic in all that he does. He always reads original scientific papers, experiments and studies instead of second-third-fourth-hand reports about the 'virus' in the media which are repeating the repeated repetition of the narrative. When he did so with the original Chinese 'virus' papers Kaufman

realised that there was no evidence of a 'SARS-Cov-2'. They had never – from the start – shown it to exist and every repeat of this claim worldwide was based on the accepted existence of proof that was nowhere to be found – see Kary Mullis and HIV. Here we go again.

Let's postulate

Kaufman discovered that the Chinese authorities immediately concluded that the cause of an illness that broke out among about 200 initial patients in Wuhan was a 'new virus' when there were no grounds to make that conclusion. The alleged 'virus' was not isolated from other genetic material in their samples and then shown through a system known as Koch's postulates to be the causative agent of the illness. The world was told that the SARS-Cov-2 'virus' caused a disease they called 'Covid-19' which had 'flu-like' symptoms and could lead to respiratory problems and pneumonia. If it wasn't so tragic it would almost be funny. *'Flu-like' symptoms'? Pneumonia? Respiratory disease?* What in *CHINA* and particularly in *Wuhan*, one of the most polluted cities in the world with a resulting epidemic of respiratory disease?? Three hundred thousand people get pneumonia in China every year and there are nearly a billion cases worldwide of 'flu-like symptoms'. These have a whole range of causes – including pollution in Wuhan – but no other possibility was credibly considered in late 2019 when the world was told there was a new and deadly 'virus'. The global prevalence of pneumonia and 'flu-like systems' gave the Cult networks unlimited potential to re-diagnose these other causes as the mythical 'Covid-19' and that is what they did from the very start. Kaufman revealed how Chinese medical and science authorities (all subordinates to the Cult-owned communist government) took genetic material from the lungs of only a few of the first patients. The material contained their own cells, bacteria, fungi and other microorganisms living in their bodies. The only way you could prove the existence of the 'virus' and its responsibility for the alleged 'Covid-19' was to isolate the virus from all the other material – a process also known as 'purification' – and

then follow the postulates sequence developed in the late 19th century by German physician and bacteriologist Robert Koch which became the 'gold standard' for connecting an alleged causation agent to a disease:

1. The microorganism (bacteria, fungus, virus, etc.) must be present in every case of the disease and all patients must have the same symptoms. It must also *not be present in healthy individuals*.

2. The microorganism must be isolated from the host with the disease. If the microorganism is a bacteria or fungus it must be grown in a pure culture. If it is a virus, it must be purified (i.e. containing no other material except the virus particles) from a clinical sample.

3. The specific disease, with all of its characteristics, must be reproduced when the infectious agent (the purified virus or a pure culture of bacteria or fungi) is inoculated into a healthy, susceptible host.

4. The microorganism must be recoverable from the experimentally infected host as in step 2.

Not one of these criteria has been met in the case of 'SARS-Cov-2' and 'Covid-19'. Not ONE. *EVER*. Robert Koch refers to bacteria and not viruses. What are called 'viral particles' are so minute (hence masks are useless by any definition) that they could only be seen after the invention of the electron microscope in the 1930s and can still only be observed through that means. American bacteriologist and virologist Thomas Milton Rivers, the so-called 'Father of Modern Virology' who was very significantly director of the Rockefeller Institute for Medical Research in the 1930s, developed a less stringent version of Koch's postulates to identify 'virus' causation known as 'Rivers criteria'. 'Covid' did not pass that process either. Some even doubt whether any 'virus' can be isolated from other particles containing genetic material in the Koch method. Freedom of Information requests in many countries asking for scientific proof that the 'Covid virus' has been purified and isolated and shown to exist have all come back with a 'we don't have that' and when this happened with a request to the UK Department of Health they added this comment:

However, outside of the scope of the [Freedom of Information Act] and on a discretionary basis, the following information has been advised to us, which may be of interest. Most infectious diseases are caused by viruses, bacteria or fungi. Some bacteria or fungi have the capacity to grow on their own in isolation, for example in colonies on a petri dish. Viruses are different in that they are what we call 'obligate pathogens' – that is, they cannot survive or reproduce without infecting a host ...

… For some diseases, it is possible to establish causation between a microorganism and a disease by isolating the pathogen from a patient, growing it in pure culture and reintroducing it to a healthy organism. These are known as 'Koch's postulates' and were developed in 1882. However, as our understanding of disease and different disease-causing agents has advanced, these are no longer the method for determining causation [Andrew Kaufman asks why in that case are there two published articles falsely claiming to satisfy Koch's postulates].

It has long been known that viral diseases cannot be identified in this way as viruses cannot be grown in 'pure culture'. When a patient is tested for a viral illness, this is normally done by looking for the presence of antigens, or viral genetic code in a host with molecular biology techniques [Kaufman asks how you could know the origin of these chemicals without having a pure culture for comparison].

For the record 'antigens' are defined so:

Invading microorganisms have antigens on their surface that the human body can recognise as being foreign – meaning not belonging to it. When the body recognises a foreign antigen, lymphocytes (white blood cells) produce antibodies, which are complementary in shape to the antigen.

Notwithstanding that this is open to question in relation to 'SARS-Cov-2' the presence of 'antibodies' can have many causes and they are found in people that are perfectly well. Kary Mullis said: 'Antibodies … had always been considered evidence of past disease, not present disease.'

'Covid' really is a *computer* 'virus'

Where the UK Department of Health statement says 'viruses' are now 'diagnosed' through a 'viral genetic code in a host with molecular biology techniques', they mean … *the PCR test* which its inventor said cannot test for infectious disease. They have no credible method of connecting a 'virus' to a disease and we will see that there is no scientific proof that any 'virus' causes any disease or there is any such thing as a 'virus' in the way that it is described. Tenacious Canadian researcher Christine Massey and her team made

some 40 Freedom of Information requests to national public health agencies in different countries asking for proof that SARS-CoV-2 has been isolated and not one of them could supply that information. Massey said of her request in Canada: 'Freedom of Information reveals Public Health Agency of Canada has no record of 'SARS-COV-2' isolation performed by anyone, anywhere, ever.' If you accept the comment from the UK Department of Health it's because they can't isolate a 'virus'. Even so many 'science' papers claimed to have isolated the 'Covid virus' until they were questioned and had to admit they hadn't. A reply from the Robert Koch Institute in Germany was typical: 'I am not aware of a paper which purified isolated SARS-CoV-2.' So what the hell was Christian Drosten and his gang using to design the 'Covid' testing protocol that has produced all the illusory Covid' cases and 'Covid' deaths when the head of the Chinese version of the CDC admitted there was a problem right from the start in that the 'virus' had never been isolated/purified? Breathe deeply: What they are calling 'Covid' is actually created by a *computer program* i.e. *they made it up* – er, that's it. They took lung fluid, with many sources of genetic material, from one single person alleged to be infected with Covid-19 by a PCR test which they *claimed*, without clear evidence, contained a 'virus'. They used several computer programs to create a model of a theoretical virus genome sequence from more than fifty-six million small sequences of RNA, each of an unknown source, assembling them like a puzzle with no known solution. The computer filled in the gaps with sequences from bits in the gene bank to make it look like a bat SARS-like coronavirus! A wave of the magic wand and poof, an in silico (computer-generated) genome, a scientific fantasy, was created. UK health researcher Dr Kevin Corbett made the same point with this analogy:

> … It's like giving you a few bones and saying that's your fish. It could be any fish. Not even a skeleton. Here's a few fragments of bones. That's your fish … It's all from gene bank and the bits of the virus sequence that weren't there they made up.
>
> They synthetically created them to fill in the blanks. That's what genetics is; it's a code. So it's ABBBCCDDD and you're missing some what you think is EEE so you put it in. It's all

synthetic. You just manufacture the bits that are missing. This is the end result of the geneticization of virology. This is basically a computer virus.

Further confirmation came in an email exchange between British citizen journalist Frances Leader and the government's Medicines & Healthcare Products Regulatory Agency (the Gates-funded MHRA) which gave emergency permission for untested 'Covid vaccines' to be used. The agency admitted that the 'vaccine' is not based on an isolated 'virus', but comes from a *computer-generated model*. Frances Leader was naturally banned from Cult-owned fascist Twitter for making this exchange public. The process of creating computer-generated alleged 'viruses' is called 'in silico' or 'in silicon' – computer chips – and the term 'in silico' is believed to originate with biological experiments using only a computer in 1989. 'Vaccines' involved with 'Covid' are also produced 'in silico' or by computer not a natural process. If the original 'virus' is nothing more than a made-up computer model how can there be 'new variants' of something that never existed in the first place? They are not new 'variants'; they are new *computer models* only minutely different to the original program and designed to further terrify the population into having the 'vaccine' and submitting to fascism. You want a 'new variant'? Click, click, enter – there you go. Tell the medical profession that you have discovered a 'South African variant', 'UK variants' or a 'Brazilian variant' and in the usual HIV-causes-AIDS manner they will unquestioningly repeat it with no evidence whatsoever to support these claims. They will go on television and warn about the dangers of 'new variants' while doing nothing more than repeating what they have been told to be true and knowing that any deviation from that would be career suicide. Big-time insiders will know it's a hoax, but much of the medical community is clueless about the way they are being played and themselves play the public without even being aware they are doing so. What an interesting 'coincidence' that AstraZeneca and Oxford University were conducting 'Covid vaccine trials' in the three countries – the UK, South Africa and Brazil – where the first three 'variants' were claimed to have 'broken out'.

Here's your 'virus' – it's a unicorn

Dr Andrew Kaufman presented a brilliant analysis describing how the 'virus' was imagined into fake existence when he dissected an article published by *Nature* and written by 19 authors detailing *alleged* 'sequencing of a complete viral genome' of the 'new SARS-CoV-2 virus'. This computer-modelled *in silico* genome was used as a template for all subsequent genome sequencing experiments that resulted in the so-called variants which he said now number more than *6,000*. The fake genome was constructed from more than 56 million individual short strands of RNA. Those little pieces were assembled into longer pieces by finding areas of overlapping sequences. The computer programs created over two million possible combinations from which the authors simply chose the longest one. They then compared this to a 'bat virus' and the computer 'alignment' rearranged the sequence and filled in the gaps! They called this computer-generated abomination the 'complete genome'. Dr Tom Cowan, a fellow medical author and collaborator with Kaufman, said such computer-generation constitutes scientific fraud and he makes this superb analogy:

> Here is an equivalency: A group of researchers claim to have found a unicorn because they found a piece of a hoof, a hair from a tail, and a snippet of a horn. They then add that information into a computer and program it to re-create the unicorn, and they then claim this computer re-creation is the real unicorn. Of course, they had never actually seen a unicorn so could not possibly have examined its genetic makeup to compare their samples with the actual unicorn's hair, hooves and horn.
>
> The researchers claim they decided which is the real genome of SARS-CoV-2 by 'consensus', sort of like a vote. Again, different computer programs will come up with different versions of the imaginary 'unicorn', so they come together as a group and decide which is the real imaginary unicorn.

This is how the 'virus' that has transformed the world was brought into fraudulent 'existence'. Extraordinary, yes, but as the Nazis said the bigger the lie the more will believe it. Cowan, however, wasn't finished and he went on to identify what he called the real blockbuster in the paper. He quotes this section from a paper written

by virologists and published by the CDC and then explains what it means:

Therefore, we examined the capacity of SARS-CoV-2 to infect and replicate in several common primate and human cell lines, including human adenocarcinoma cells (A549), human liver cells (HUH 7.0), and human embryonic kidney cells (HEK-293T). In addition to Vero E6 and Vero CCL81 cells. ... Each cell line was inoculated at high multiplicity of infection and examined 24h post-infection.

No CPE was observed in any of the cell lines except in Vero cells, which grew to greater than 10 to the 7th power at 24 h post-infection. In contrast, HUH 7.0 and 293T showed only modest viral replication, and A549 cells were incompatible with SARS CoV-2 infection.

Cowan explains that when virologists attempt to prove infection they have three possible 'hosts' or models on which they can test. The first was humans. Exposure to humans was generally not done for ethical reasons and has never been done with SARS-CoV-2 or any coronavirus. The second possible host was animals. Cowan said that forgetting for a moment that they never actually use purified virus when exposing animals they do use solutions that they *claim* contain the virus. Exposure to animals has been done with SARS-CoV-2 in an experiment involving mice and this is what they found: *None of the wild (normal) mice got sick*. In a group of genetically-modified mice, a statistically insignificant number lost weight and had slightly bristled fur, but they experienced nothing like the illness called 'Covid-19'. Cowan said the third method – the one they mostly rely on – is to inoculate solutions they *say* contain the virus onto a variety of tissue cultures. This process had never been shown to kill tissue *unless* the sample material was starved of nutrients and poisoned as *part of the process*. Yes, incredibly, in tissue experiments designed to show the 'virus' is responsible for killing the tissue they starve the tissue of nutrients and add toxic drugs including antibiotics and they do not have control studies to see if it's the starvation and poisoning that is degrading the tissue rather than the 'virus' they allege to be in there somewhere. You want me to pinch you? Yep, I understand. Tom Cowan said this about the whole nonsensical farce as he explains what that quote from the CDC paper really means:

The shocking thing about the above quote is that using their own methods, the virologists found that solutions containing SARS-CoV-2 – even in high amounts – were NOT, I repeat NOT, infective to any of the three human tissue cultures they tested. In plain English, this means they proved, on their terms, that this 'new coronavirus' is not infectious to human beings. It is ONLY infective to monkey kidney cells, and only then when you add two potent drugs (gentamicin and amphotericin), known to be toxic to kidneys, to the mix.

My friends, read this again and again. These virologists, published by the CDC, performed a clear proof, on their terms, showing that the SARS-CoV-2 virus is harmless to human beings. That is the only possible conclusion, but, unfortunately, this result is not even mentioned in their conclusion. They simply say they can provide virus stocks cultured only on monkey Vero cells, thanks for coming.

Cowan concluded: 'If people really understood how this "science" was done, I would hope they would storm the gates and demand honesty, transparency and truth.' Dr Michael Yeadon, former Vice President and Chief Scientific Adviser at drug giant Pfizer has been a vocal critic of the 'Covid vaccine' and its potential for multiple harm. He said in an interview in April, 2021, that 'not one [vaccine] has the virus. He was asked why vaccines normally using a 'dead' version of a disease to activate the immune system were not used for 'Covid' and instead we had the synthetic methods of the 'mRNA Covid vaccine'. Yeadon said that to do the former 'you'd have to have some of [the virus] wouldn't you?' He added: 'No-one's got any – seriously.' Yeadon said that surely they couldn't have fooled the whole world for a year without having a virus, 'but oddly enough ask around – no one's got it'. He didn't know why with all the 'great labs' around the world that the virus had not been isolated – 'Maybe they've been too busy running bad PCR tests and vaccines that people don't need.' What is today called 'science' is not 'science' at all. Science is no longer what is, but whatever people can be manipulated to *believe* that it is. Real science has been hijacked by the Cult to dispense and produce the 'expert scientists' and contentions that suit the agenda of the Cult. How big-time this has happened with the 'Covid' hoax which is entirely based on fake science delivered by fake 'scientists' and fake 'doctors'. The human-caused climate change hoax is also entirely based on fake science delivered by fake 'scientists' and fake 'climate experts'. In both cases real

scientists, climate experts and doctors have their views suppressed and deleted by the Cult-owned science establishment, media and Silicon Valley. This is the 'science' that politicians claim to be 'following' and a common denominator of 'Covid' and climate are Cult psychopaths Bill Gates and his mate Klaus Schwab at the Gates-funded World Economic Forum. But, don't worry, it's all just a coincidence and absolutely nothing to worry about. *Zzzzzzzz*.

What is a 'virus' REALLY?

Dr Tom Cowan is one of many contesting the very existence of viruses let alone that they cause disease. This is understandable when there is no scientific evidence for a disease-causing 'virus'. German virologist Dr Stefan Lanka won a landmark case in 2017 in the German Supreme Court over his contention that there is no such thing as a measles virus. He had offered a big prize for anyone who could prove there is and Lanka won his case when someone sought to claim the money. There is currently a prize of more than 225,000 euros on offer from an Isolate Truth Fund for anyone who can prove the isolation of SARS-CoV-2 and its genetic substance. Lanka wrote in an article headed 'The Misconception Called Virus' that scientists think a 'virus' is causing tissue to become diseased and degraded when in fact it is the *processes they are using* which do that – not a 'virus'. Lanka has done an important job in making this point clear as Cowan did in his analysis of the CDC paper. Lanka says that all claims about viruses as disease-causing pathogens are wrong and based on 'easily recognisable, understandable and verifiable misinterpretations.' Scientists believed they were working with 'viruses' in their laboratories when they were really working with 'typical particles of specific dying tissues or cells …' Lanka said that the tissue decaying process claimed to be caused by a 'virus' still happens when no alleged 'virus' is involved. It's the *process* that does the damage and not a 'virus'. The genetic sample is deprived of nutrients, removed from its energy supply through removal from the body and then doused in toxic antibiotics to remove any bacteria. He confirms again that establishment scientists do not (pinch me)

conduct control experiments to see if this is the case and if they did they would see the claims that 'viruses' are doing the damage is nonsense. He adds that during the measles 'virus' court case he commissioned an independent laboratory to perform just such a control experiment and the result was that the tissues and cells died in the exact same way as with alleged 'infected' material. This is supported by a gathering number of scientists, doctors and researchers who reject what is called 'germ theory' or the belief in the body being infected by contagious sources emitted by other people. Researchers Dawn Lester and David Parker take the same stance in their highly-detailed and sourced book *What Really Makes You Ill – Why everything you thought you knew about disease is wrong* which was recommended to me by a number of medical professionals genuinely seeking the truth. Lester and Parker say there is no provable scientific evidence to show that a 'virus' can be transmitted between people or people and animals or animals and people:

> The definition also claims that viruses are the cause of many diseases, as if this has been definitively proven. But this is not the case; there is no original scientific evidence that definitively demonstrates that any virus is the cause of any disease. The burden of proof for any theory lies with those who proposed it; but none of the existing documents provides 'proof' that supports the claim that 'viruses' are pathogens.

Dr Tom Cowan employs one of his clever analogies to describe the process by which a 'virus' is named as the culprit for a disease when what is called a 'virus' is only material released by cells detoxing themselves from infiltration by chemical or radiation poisoning. The tidal wave of technologically-generated radiation in the 'smart' modern world plus all the toxic food and drink are causing this to happen more than ever. Deluded 'scientists' misread this as a gathering impact of what they wrongly label 'viruses'.

Paper can infect houses

Cowan said in an article for davidicke.com – with his tongue only mildly in his cheek – that he believed he had made a tremendous

discovery that may revolutionise science. He had discovered that small bits of paper are alive, 'well alive-ish', can 'infect' houses, and then reproduce themselves inside the house. The result was that this explosion of growth in the paper inside the house causes the house to explode, blowing it to smithereens. His evidence for this new theory is that in the past months he had carefully examined many of the houses in his neighbourhood and found almost no scraps of paper on the lawns and surrounds of the house. There was an occasional stray label, but nothing more. Then he would return to these same houses a week or so later and with a few, not all of them, particularly the old and decrepit ones, he found to his shock and surprise they were littered with stray bits of paper. He knew then that the paper had infected these houses, made copies of itself, and blew up the house. A young boy on a bicycle at one of the sites told him he had seen a demolition crew using dynamite to explode the house the previous week, but Cowan dismissed this as the idle thoughts of silly boys because 'I was on to something big'. He was on to how 'scientists' mistake genetic material in the detoxifying process for something they call a 'virus'. Cowan said of his house and paper story:

> If this sounds crazy to you, it's because it should. This scenario is obviously nuts. But consider this admittedly embellished, for effect, current viral theory that all scientists, medical doctors and virologists currently believe.

He takes the example of the 'novel SARS-Cov2' virus to prove the point. First they take someone with an undefined illness called 'Covid-19' and don't even attempt to find any virus in their sputum. Never mind the scientists still describe how this 'virus', which they have not located attaches to a cell receptor, injects its genetic material, in 'Covid's' case, RNA, into the cell. The RNA once inserted exploits the cell to reproduce itself and makes 'thousands, nay millions, of copies of itself … Then it emerges victorious to claim its next victim':

If you were to look in the scientific literature for proof, actual scientific proof, that uniform SARS-CoV2 viruses have been properly isolated from the sputum of a sick person, that actual spike proteins could be seen protruding from the virus (which has not been found), you would find that such evidence doesn't exist.

If you go looking in the published scientific literature for actual pictures, proof, that these spike proteins or any viral proteins are ever attached to any receptor embedded in any cell membrane, you would also find that no such evidence exists. If you were to look for a video or documented evidence of the intact virus injecting its genetic material into the body of the cell, reproducing itself and then emerging victorious by budding off the cell membrane, you would find that no such evidence exists.

The closest thing you would find is electron micrograph pictures of cellular particles, possibly attached to cell debris, both of which to be seen were stained by heavy metals, a process that completely distorts their architecture within the living organism. This is like finding bits of paper stuck to the blown-up bricks, thereby proving the paper emerged by taking pieces of the bricks on its way out.

The Enders baloney

Cowan describes the 'Covid' story as being just as make-believe as his paper story and he charts back this fantasy to a Nobel Prize winner called John Enders (1897-1985), an American biomedical scientist who has been dubbed 'The Father of Modern Vaccines'. Enders is claimed to have 'discovered' the process of the viral culture which 'proved' that a 'virus' caused measles. Cowan explains how Enders did this 'by using the EXACT same procedure that has been followed by every virologist to find and characterize every new virus since 1954'. Enders took throat swabs from children with measles and immersed them in 2ml of milk. Penicillin (100u/ml) and the antibiotic streptomycin (50,g/ml) were added and the whole mix was centrifuged – rotated at high speed to separate large cellular debris from small particles and molecules as with milk and cream, for example. Cowan says that if the aim is to find little particles of genetic material ('viruses') in the snot from children with measles it would seem that the last thing you would do is mix the snot with other material – milk –that also has genetic material. 'How are you ever going to know whether whatever you found came from the snot or the milk?' He points out that streptomycin is a 'nephrotoxic' or poisonous-to-the-kidney drug. You will see the relevance of that

shortly. Cowan says that it gets worse, much worse, when Enders describes the culture medium upon which the virus 'grows': 'The culture medium consisted of bovine amniotic fluid (90%), beef embryo extract (5%), horse serum (5%), antibiotics and phenol red as an indicator of cell metabolism.' Cowan asks incredulously: 'Did he just say that the culture medium also contained fluids and tissues that are themselves rich sources of genetic material?' The genetic cocktail, or 'medium', is inoculated onto tissue and cells from rhesus monkey *kidney* tissue. This is where the importance of streptomycin comes in and currently-used antimicrobials and other drugs that are *poisonous to kidneys* and used in ALL modern viral cultures (e.g. gentamicin, streptomycin, and amphotericin). Cowan asks: 'How are you ever going to know from this witch's brew where any genetic material comes from as we now have five different sources of rich genetic material in our mix?' Remember, he says, that all genetic material, whether from monkey kidney tissues, bovine serum, milk, etc., is made from the exact same components. The same central question returns: 'How are you possibly going to know that it was the virus that killed the kidney tissue and not the toxic antibiotic and starvation rations on which you are growing the tissue?' John Enders answered the question himself – *you can't*:

A second agent was obtained from an uninoculated culture of monkey kidney cells. The cytopathic changes [death of the cells] it induced in the unstained preparations could not be distinguished with confidence from the viruses isolated from measles.

The death of the cells ('cytopathic changes') happened in exactly the same manner, whether they inoculated the kidney tissue with the measles snot or not, Cowan says. 'This is evidence that the destruction of the tissue, the very proof of viral causation of illness, was not caused by anything in the snot because they saw the same destructive effect when the snot was not even used … the cytopathic, i.e., cell-killing, changes come from the process of the culture itself, not from any virus in any snot, period.' Enders quotes in his 1957 paper a virologist called Ruckle as reporting similar findings 'and in addition has isolated an agent from monkey kidney tissue that is so

far indistinguishable from human measles virus'. In other words, Cowan says, these particles called 'measles viruses' are simply and clearly breakdown products of the starved and poisoned tissue. For measles 'virus' see all 'viruses' including the so-called 'Covid virus'. Enders, the 'Father of Modern Vaccines', also said:

There is a potential risk in employing cultures of primate cells for the production of vaccines composed of attenuated virus, since the presence of other agents possibly latent in primate tissues cannot be definitely excluded by any known method.

Cowan further quotes from a paper published in the journal *Viruses* in May, 2020, while the 'Covid pandemic' was well underway in the media if not in reality. 'EVs' here refers to particles of genetic debris from our own tissues, such as exosomes of which more in a moment: 'The remarkable resemblance between EVs and viruses has caused quite a few problems in the studies focused on the analysis of EVs released during viral infections.' Later the paper adds that to date a reliable method that can actually guarantee a complete separation (of EVs from viruses) DOES NOT EXIST. This was published at a time when a fairy tale 'virus' was claimed in total certainty to be causing a fairy tale 'viral disease' called 'Covid-19' – a fairy tale that was already well on the way to transforming human society in the image that the Cult has worked to achieve for so long. Cowan concludes his article:

To summarize, there is no scientific evidence that pathogenic viruses exist. What we think of as 'viruses' are simply the normal breakdown products of dead and dying tissues and cells. When we are well, we make fewer of these particles; when we are starved, poisoned, suffocated by wearing masks, or afraid, we make more.

There is no engineered virus circulating and making people sick. People in laboratories all over the world are making genetically modified products to make people sick. These are called vaccines. There is no virome, no 'ecosystem' of viruses, viruses are not 8%, 50% or 100 % of our genetic material. These are all simply erroneous ideas based on the misconception called a virus.

What is 'Covid'? Load of bollocks

The background described here by Cowan and Lanka was emphasised in the first video presentation that I saw by Dr Andrew Kaufman when he asked whether the 'Covid virus' was in truth a natural defence mechanism of the body called 'exosomes'. These are released by cells when in states of toxicity – see the same themes returning over and over. They are released ever more profusely as chemical and radiation toxicity increases and think of the potential effect therefore of 5G alone as its destructive frequencies infest the human energetic information field with a gathering pace (5G went online in Wuhan in 2019 as the 'virus' emerged). I'll have more about this later. Exosomes transmit a warning to the rest of the body that 'Houston, we have a problem'. Kaufman presented images of exosomes and compared them with 'Covid' under an electron microscope and the similarity was remarkable. They both attach to the same cell receptors (*claimed* in the case of 'Covid'), contain the same genetic material in the form of RNA or ribonucleic acid, and both are found in 'viral cell cultures' with damaged or dying cells. James Hildreth MD, President and Chief Executive Officer of the Meharry Medical College at Johns Hopkins, said: 'The virus is fully an exosome in every sense of the word.' Kaufman's conclusion was that there is no 'virus': 'This entire pandemic is a completely manufactured crisis … there is no evidence of anyone dying from [this] illness.' Dr Tom Cowan and Sally Fallon Morell, authors of *The Contagion Myth*, published a statement with Dr Kaufman in February, 2021, explaining why the 'virus' does not exist and you can read it that in full in the Appendix.

'Virus' theory can be traced to the 'cell theory' in 1858 of German physician Rudolf Virchow (1821-1920) who contended that disease originates from a single cell infiltrated by a 'virus'. Dr Stefan Lanka said that findings and insights with respect to the structure, function and central importance of tissues in the creation of life, which were already known in 1858, comprehensively refute the cell theory. Virchow ignored them. We have seen the part later played by John Enders in the 1950s and Lanka notes that infection theories were only established as a global dogma through the policies and

eugenics of the Third Reich in Nazi Germany (creation of the same Sabbatian cult behind the 'Covid' hoax). Lanka said: 'Before 1933, scientists dared to contradict this theory; after 1933, these critical scientists were silenced'. Dr Tom Cowan's view is that ill-heath is caused by too much of something, too little of something, or toxification from chemicals and radiation – not contagion. We must also highlight as a major source of the 'virus' theology a man still called the 'Father of Modern Virology' – Thomas Milton Rivers (1888-1962). There is no way given the Cult's long game policy that it was a coincidence for the 'Father of Modern Virology' to be director of the Rockefeller Institute for Medical Research from 1937 to 1956 when he is credited with making the Rockefeller Institute a leader in 'viral research'. Cult Rockefellers were the force behind the creation of Big Pharma 'medicine', established the World Health Organisation in 1948, and have long and close associations with the Gates family that now runs the WHO during the pandemic hoax through mega-rich Cult gofer and psychopath Bill Gates.

Only a Renegade Mind can see through all this bullshit by asking the questions that need to be answered, not taking 'no' or prevarication for an answer, and certainly not hiding from the truth in fear of speaking it. Renegade Minds have always changed the world for the better and they will change this one no matter how bleak it may currently appear to be.

CHAPTER SIX

Sequence of deceit

If you tell the truth, you don't have to remember anything
Mark Twain

Against the background that I have laid out this far the sequence that took us from an invented 'virus' in Cult-owned China in late 2019 to the fascist transformation of human society can be seen and understood in a whole new context.

We were told that a deadly disease had broken out in Wuhan and the world media began its campaign (coordinated by behavioural psychologists as we shall see) to terrify the population into unquestioning compliance. We were shown images of Chinese people collapsing in the street which never happened in the West with what was supposed to be the same condition. In the earliest days when alleged cases and deaths were few the fear register was hysterical in many areas of the media and this would expand into the common media narrative across the world. The real story was rather different, but we were never told that. The Chinese government, one of the Cult's biggest centres of global operation, said they had discovered a new illness with flu-like and pneumonia-type symptoms in a city with such toxic air that it is overwhelmed with flu-like symptoms, pneumonia and respiratory disease. Chinese scientists said it was a new – 'novel' – coronavirus which they called Sars-Cov-2 and that it caused a disease they labelled 'Covid-19'. There was no evidence for this and the 'virus' has never to this day been isolated, purified and its genetic code established from that. It

was from the beginning a computer-generated fiction. Stories of Chinese whistleblowers saying the number of deaths was being supressed or that the 'new disease' was related to the Wuhan bio-lab misdirected mainstream and alternative media into cul-de-sacs to obscure the real truth – there was no 'virus'.

Chinese scientists took genetic material from the lung fluid of just a few people and said they had found a 'new' disease when this material had a wide range of content. There was no evidence for a 'virus' for the very reasons explained in the last two chapters. The 'virus' has never been shown to (a) exist and (b) cause any disease. People were diagnosed on symptoms that are so widespread in Wuhan and polluted China and with a PCR test that can't detect infectious disease. On this farce the whole global scam was sold to the rest of the world which would also diagnose respiratory disease as 'Covid-19' from symptoms alone or with a PCR test not testing for a 'virus'. Flu miraculously disappeared *worldwide* in 2020 and into 2021 as it was redesignated 'Covid-19'. It was really the same old flu with its 'flu-like' symptoms attributed to 'flu-like' 'Covid-19'. At the same time with very few exceptions the Chinese response of draconian lockdown and fascism was the chosen weapon to respond across the West as recommended by the Cult-owned Tedros at the Cult-owned World Health Organization run by the Cult-owned Gates. All was going according to plan. Chinese scientists – everything in China is controlled by the Cult-owned government – compared their contaminated RNA lung-fluid material with other RNA sequences and said it appeared to be just under 80 percent identical to the SARS-CoV-1 'virus' claimed to be the cause of the SARS (severe acute respiratory syndrome) 'outbreak' in 2003. They decreed that because of this the 'new virus' had to be related and they called it SARS-CoV-2. There are some serious problems with this assumption and *assumption* was all it was. Most 'factual' science turns out to be assumptions repeated into everyone-knows-that. A match of under 80-percent is meaningless. Dr Kaufman makes the point that there's a *96 percent* genetic correlation between humans and chimpanzees, but 'no one would say our genetic material is part

of the chimpanzee family'. Yet the Chinese authorities were claiming that a much lower percentage, less than 80 percent, proved the existence of a new 'coronavirus'. For goodness sake human DNA is 60 percent similar to a *banana*.

You are feeling sleepy

The entire 'Covid' hoax is a global Psyop, a psychological operation to program the human mind into believing and fearing a complete fantasy. A crucial aspect of this was what *appeared* to happen in Italy. It was all very well streaming out daily images of an alleged catastrophe in Wuhan, but to the Western mind it was still on the other side of the world in a very different culture and setting. A reaction of 'this could happen to me and my family' was still nothing like as intense enough for the mind-doctors. The Cult needed a Western example to push people over that edge and it chose Italy, one of its major global locations going back to the Roman Empire. An Italian 'Covid' crisis was manufactured in a particular area called Lombardy which just happens to be notorious for its toxic air and therefore respiratory disease. Wuhan, China, déjà vu. An hysterical media told horror stories of Italians dying from 'Covid' in their droves and how Lombardy hospitals were being overrun by a tidal wave of desperately ill people needing treatment after being struck down by the 'deadly virus'. Here was the psychological turning point the Cult had planned. Wow, if this is happening in Italy, the Western mind concluded, this indeed could happen to me and my family. Another point is that Italian authorities responded by following the Chinese blueprint so vehemently recommended by the Cult-owned World Health Organization. They imposed fascistic lockdowns on the whole country viciously policed with the help of surveillance drones sweeping through the streets seeking out anyone who escaped from mass house arrest. Livelihoods were destroyed and psychology unravelled in the way we have witnessed since in all lockdown countries. Crucial to the plan was that Italy responded in this way to set the precedent of suspending freedom and imposing fascism in a 'Western liberal democracy'. I emphasised in an

animated video explanation on davidicke.com posted in the summer of 2020 how important it was to the Cult to expand the Chinese lockdown model across the West. Without this, and the bare-faced lie that non-symptomatic people could still transmit a 'disease' they didn't have, there was no way locking down the whole population, sick and not sick, could be pulled off. At just the right time and with no evidence Cult operatives and gofers claimed that people without symptoms could pass on the 'disease'. In the name of protecting the 'vulnerable' like elderly people, who lockdowns would kill by the tens of thousands, we had for the first time healthy people told to isolate as well as the sick. The great majority of people who tested positive had no symptoms because there was nothing wrong with them. It was just a trick made possible by a test not testing for the 'virus'.

Months after my animated video the Gates-funded Professor Neil Ferguson at the Gates-funded Imperial College confirmed that I was right. He didn't say it in those terms, naturally, but he did say it. Ferguson will enter the story shortly for his outrageously crazy 'computer models' that led to Britain, the United States and many other countries following the Chinese and now Italian methods of response. Put another way, following the Cult script. Ferguson said that SAGE, the UK government's scientific advisory group which has controlled 'Covid' policy from the start, wanted to follow the Chinese lockdown model (while they all continued to work and be paid), but they wondered if they could possibly, in Ferguson's words, 'get away with it in Europe'. 'Get away with it'? Who the hell do these moronic, arrogant people think they are? This appalling man Ferguson said that once Italy went into national lockdown they realised they, too, could mimic China:

> It's a communist one-party state, we said. We couldn't get away with it in Europe, we thought … and then Italy did it. And we realised we could. Behind this garbage from Ferguson is a simple fact: Doing the same as China in every country was the plan from the start and Ferguson's 'models' would play a central role in achieving that. It's just a coincidence, of course, and absolutely nothing to worry your little head about.

Oops, sorry, our mistake

Once the Italian segment of the Psyop had done the job it was designed to do a very different story emerged. Italian authorities revealed that 99 percent of those who had 'died from Covid-19' in Italy had one, two, three, or more 'co-morbidities' or illnesses and health problems that could have ended their life. The US Centers for Disease Control and Prevention (CDC) published a figure of 94 percent for Americans dying of 'Covid' while having other serious medical conditions – on average two to three (some five or six) other potential causes of death. In terms of death from an unproven 'virus' I say it is 100 percent. The other one percent in Italy and six percent in the US would presumably have died from 'Covid's' flu-like symptoms with a range of other possible causes in conjunction with a test not testing for the 'virus'. Fox News reported that even more startling figures had emerged in one US county in which 410 of 422 deaths attributed to 'Covid-19' had other potentially deadly health conditions. The Italian National Health Institute said later that the average age of people dying with a 'Covid-19' diagnosis in Italy was about 81. Ninety percent were over 70 with ten percent over 90. In terms of other reasons to die some 80 percent had two or more chronic diseases with half having three or more including cardiovascular problems, diabetes, respiratory problems and cancer. Why is the phantom 'Covid-19' said to kill overwhelmingly old people and hardly affect the young? Old people continually die of many causes and especially respiratory disease which you can re-diagnose 'Covid-19' while young people die in tiny numbers by comparison and rarely of respiratory disease. Old people 'die of Covid' because they die of other things that can be redesignated 'Covid' and it really is that simple.

Flu has flown

The blueprint was in place. Get your illusory 'cases' from a test not testing for the 'virus' and redesignate other causes of death as 'Covid-19'. You have an instant 'pandemic' from something that is nothing more than a computer-generated fiction. With near-on a

billion people having 'flu-like' symptoms every year the potential was limitless and we can see why flu quickly and apparently miraculously disappeared *worldwide* by being diagnosed 'Covid-19'. The painfully bloody obvious was explained away by the childlike media in headlines like this in the UK '*Independent*': 'Not a single case of flu detected by Public Health England this year as Covid restrictions suppress virus'. I kid you not. The masking, social distancing and house arrest that did not make the 'Covid virus' disappear somehow did so with the 'flu virus'. Even worse the article, by a bloke called Samuel Lovett, suggested that maybe the masking, sanitising and other 'Covid' measures should continue to keep the flu away. With a ridiculousness that disturbs your breathing (it's 'Covid-19') the said Lovett wrote: 'With widespread social distancing and mask-wearing measures in place throughout the UK, the usual routes of transmission for influenza have been blocked.' He had absolutely no evidence to support that statement, but look at the consequences of him acknowledging the obvious. With flu not disappearing at all and only being relabelled 'Covid-19' he would have to contemplate that 'Covid' was a hoax on a scale that is hard to imagine. You need guts and commitment to truth to even go there and that's clearly something Samuel Lovett does not have in abundance. He would never have got it through the editors anyway.

Tens of thousands die in the United States alone every winter from flu including many with pneumonia complications. CDC figures record *45 million* Americans diagnosed with flu in 2017-2018 of which 61,000 died and some reports claim 80,000. Where was the same hysteria then that we have seen with 'Covid-19'? Some 250,000 Americans are admitted to hospital with pneumonia every year with about 50,000 cases proving fatal. About 65 million suffer respiratory disease every year and three million deaths makes this the third biggest cause of death worldwide. You only have to redesignate a portion of all these people 'Covid-19' and you have an instant global pandemic or the *appearance* of one. Why would doctors do this? They are told to do this and all but a few dare not refuse those who must be obeyed. Doctors in general are not researching their own

knowledge and instead take it direct and unquestioned from the authorities that own them and their careers. The authorities say they must now diagnose these symptoms 'Covid-19' and not flu, or whatever, and they do it. Dark suits say put 'Covid-19' on death certificates no matter what the cause of death and the doctors do it. Renegade Minds don't fall for the illusion that doctors and medical staff are all highly-intelligent, highly-principled, seekers of medical truth. *Some are*, but not the majority. They are repeaters, gofers, and yes sir, no sir, purveyors of what the system demands they purvey. The 'Covid' con is not merely confined to diseases of the lungs. Instructions to doctors to put 'Covid-19' on death certificates for anyone dying of *anything* within 28 days (or much more) of a positive test not testing for the 'virus' opened the floodgates. The term dying *with* 'Covid' and not *of* 'Covid' was coined to cover the truth. Whether it was a *with* or an *of* they were all added to the death numbers attributed to the 'deadly virus' compiled by national governments and globally by the Gates-funded Johns Hopkins operation in the United States that was so involved in those 'pandemic' simulations. Fraudulent deaths were added to the ever-growing list of fraudulent 'cases' from false positives from a false test. No wonder Professor Walter Ricciardi, scientific advisor to the Italian minister of health, said after the Lombardy hysteria had done its job that 'Covid' death rates were due to Italy having the second oldest population in the world and to *how hospitals record deaths*:

The way in which we code deaths in our country is very generous in the sense that all the people who die in hospitals with the coronavirus are deemed to be dying of the coronavirus. On re-evaluation by the National Institute of Health, only 12 per cent of death certificates have shown a direct causality from coronavirus, while 88 per cent of patients who have died have at least one pre-morbidity – many had two or three.

This is extraordinary enough when you consider the propaganda campaign to use Italy to terrify the world, but how can they even say twelve percent were genuine when the 'virus' has not been shown to exist, its 'code' is a computer program, and diagnosis comes from a test not testing for it? As in China, and soon the world, 'Covid-19' in

Italy was a redesignation of diagnosis. Lies and corruption were to become the real 'pandemic' fuelled by a pathetically-compliant medical system taking its orders from the tiny few at the top of their national hierarchy who answered to the World Health Organization which answers to Gates and the Cult. Doctors were told – ordered – to diagnose a particular set of symptoms 'Covid-19' and put that on the death certificate for any cause of death if the patient had tested positive with a test not testing for the virus or had 'Covid' symptoms like the flu. The United States even introduced big financial incentives to manipulate the figures with hospitals receiving £4,600 from the Medicare system for diagnosing someone with regular pneumonia, $13,000 if they made the diagnosis from the same symptoms 'Covid-19' pneumonia, and $39, 000 if they put a 'Covid' diagnosed patient on a ventilator that would almost certainly kill them. A few – painfully and pathetically few – medical whistleblowers revealed (before Cult-owned YouTube deleted their videos) that they had been instructed to 'let the patient crash' and put them straight on a ventilator instead of going through a series of far less intrusive and dangerous methods as they would have done before the pandemic hoax began and the financial incentives kicked in. We are talking cold-blooded murder given that ventilators are so damaging to respiratory systems they are usually the last step before heaven awaits. Renegade Minds never fall for the belief that people in white coats are all angels of mercy and cannot be full-on psychopaths. I have explained in detail in *The Answer* how what I am describing here played out across the world coordinated by the World Health Organization through the medical hierarchies in almost every country.

Medical scientist calls it

Information about the non-existence of the 'virus' began to emerge for me in late March, 2020, and mushroomed after that. I was sent an email by Sir Julian Rose, a writer, researcher, and organic farming promotor, from a medical scientist friend of his in the United States. Even at that early stage in March the scientist was able to explain

how the 'Covid' hoax was being manipulated. He said there were no reliable tests for a specific 'Covid-19 virus' and nor were there any reliable agencies or media outlets for reporting numbers of actual 'Covid-19' cases. We have seen in the long period since then that he was absolutely right. 'Every action and reaction to Covid-19 is based on totally flawed data and we simply cannot make accurate assessments,' he said. Most people diagnosed with 'Covid-19' were showing nothing more than cold and flu-like symptoms 'because most coronavirus strains *are* nothing more than cold/flu-like symptoms'. We had farcical situations like an 84-year-old German man testing positive for 'Covid-19' and his nursing home ordered to quarantine only for him to be found to have a common cold. The scientist described back then why PCR tests and what he called the 'Mickey Mouse test kits' were useless for what they were claimed to be identifying. 'The idea these kits can isolate a specific virus like Covid-19 is nonsense,' he said. Significantly, he pointed out that 'if you want to create a totally false panic about a totally false pandemic – pick a coronavirus'. This is exactly what the Cult-owned Gates, World Economic Forum and Johns Hopkins University did with their Event 201 'simulation' followed by their real-life simulation called the 'pandemic'. The scientist said that all you had to do was select the sickest of people with respiratory-type diseases in a single location – 'say Wuhan' – and administer PCR tests to them. You can then claim that anyone showing 'viral sequences' similar to a coronavirus 'which will inevitably be quite a few' is suffering from a 'new' disease:

> Since you already selected the sickest flu cases a fairly high proportion of your sample will go on to die. You can then say this 'new' virus has a CFR [case fatality rate] higher than the flu and use this to infuse more concern and do more tests which will of course produce more 'cases', which expands the testing, which produces yet more 'cases' and so on and so on. Before long you have your 'pandemic', and all you have done is use a simple test kit trick to convert the worst flu and pneumonia cases into something new that doesn't ACTUALLY EXIST [my emphasis].

He said that you then 'just run the same scam in other countries' and make sure to keep the fear message running high 'so that people

will feel panicky and less able to think critically'. The only problem to overcome was the fact *there is no* actual new deadly pathogen and only regular sick people. This meant that deaths from the 'new deadly pathogen' were going to be way too low for a real new deadly virus pandemic, but he said this could be overcome in the following ways – all of which would go on to happen:

1. You can claim this is just the beginning and more deaths are imminent [you underpin this with fantasy 'computer projections']. Use this as an excuse to quarantine everyone and then claim the quarantine prevented the expected millions of dead.

2. You can [say that people] 'minimizing' the dangers are irresponsible and bully them into not talking about numbers.

3. You can talk crap about made up numbers hoping to blind people with pseudoscience.

4. You can start testing well people (who, of course, will also likely have shreds of coronavirus [RNA] in them) and thus inflate your 'case figures' with 'asymptomatic carriers' (you will of course have to spin that to sound deadly even though any virologist knows the more symptom-less cases you have the less deadly is your pathogen).

The scientist said that if you take these simple steps 'you can have your own entirely manufactured pandemic up and running in weeks'. His analysis made so early in the hoax was brilliantly prophetic of what would actually unfold. Pulling all the information together in these recent chapters we have this is simple 1, 2, 3, of how you can delude virtually the entire human population into believing in a 'virus' that doesn't exist:

- A 'Covid case' is someone who tests positive with a test not testing for the 'virus'.

- A 'Covid death' is someone who dies of *any cause* within 28 days (or much longer) of testing positive with a test not testing for the 'virus.

- Asymptomatic means there is nothing wrong with you, but they claim you can pass on what you don't have to justify locking

down (quarantining) healthy people in totality.

The foundations of the hoax are that simple. A study involving ten million people in Wuhan, published in November, 2020, demolished the whole lie about those without symptoms passing on the 'virus'. They found '300 asymptomatic cases' and traced their contacts to find that not one of them was detected with the 'virus'. 'Asymptomatic' patients and their contacts were isolated for no less than two weeks and nothing changed. I know it's all crap, but if you are going to claim that those without symptoms can transmit 'the virus' then you must produce evidence for that and they never have. Even World Health Organization official Dr Maria Van Kerkhove, head of the emerging diseases and zoonosis unit, said as early as June, 2020, that she doubted the validity of asymptomatic transmission. She said that 'from the data we have, it still seems to be rare that an asymptomatic person actually transmits onward to a secondary individual' and by 'rare' she meant that she couldn't cite any case of asymptomatic transmission.

The Ferguson factor

The problem for the Cult as it headed into March, 2020, when the script had lockdown due to start, was that despite all the manipulation of the case and death figures they still did not have enough people alleged to have died from 'Covid' to justify mass house arrest. This was overcome in the way the scientist described: 'You can claim this is just the beginning and more deaths are imminent … Use this as an excuse to quarantine everyone and then claim the quarantine prevented the expected millions of dead.' Enter one Professor Neil Ferguson, the Gates-funded 'epidemiologist' at the Gates-funded Imperial College in London. Ferguson is Britain's Christian Drosten in that he has a dire record of predicting health outcomes, but is still called upon to advise government on the next health outcome when another 'crisis' comes along. This may seem to be a strange and ridiculous thing to do. Why would you keep turning for policy guidance to people who have a history of being

monumentally wrong? Ah, but it makes sense from the Cult point of view. These 'experts' keep on producing predictions that suit the Cult agenda for societal transformation and so it was with Neil Ferguson as he revealed his horrific (and clearly insane) computer model predictions that allowed lockdowns to be imposed in Britain, the United States and many other countries. Ferguson does not have even an A-level in biology and would appear to have no formal training in computer modelling, medicine or epidemiology, according to Derek Winton, an MSc in Computational Intelligence. He wrote an article somewhat aghast at what Ferguson did which included taking no account of respiratory disease 'seasonality' which means it is far worse in the winter months. Who would have thought that respiratory disease could be worse in the winter? Well, certainly not Ferguson.

The massively China-connected Imperial College and its bizarre professor provided the excuse for the long-incubated Chinese model of human control to travel westward at lightning speed. Imperial College confirms on its website that it collaborates with the Chinese Research Institute; publishes more than 600 research papers every year with Chinese research institutions; has 225 Chinese staff; 2,600 Chinese students – the biggest international group; 7,000 former students living in China which is the largest group outside the UK; and was selected for a tour by China's President Xi Jinping during his state visit to the UK in 2015. The college takes major donations from China and describes itself as the UK's number one university collaborator with Chinese research institutions. The China communist/fascist government did not appear phased by the woeful predictions of Ferguson and Imperial when during the lockdown that Ferguson induced the college signed a five-year collaboration deal with China tech giant Huawei that will have Huawei's indoor 5G network equipment installed at the college's West London tech campus along with an 'AI cloud platform'. The deal includes Chinese sponsorship of Imperial's Venture Catalyst entrepreneurship competition. Imperial is an example of the enormous influence the Chinese government has within British and North American

universities and research centres – and further afield. Up to 200 academics from more than a dozen UK universities are being investigated on suspicion of 'unintentionally' helping the Chinese government build weapons of mass destruction by 'transferring world-leading research in advanced military technology such as aircraft, missile designs and cyberweapons'. Similar scandals have broken in the United States, but it's all a coincidence. Imperial College serves the agenda in many other ways including the promotion of every aspect of the United Nations Agenda 21/2030 (the Great Reset) and produced computer models to show that human-caused 'climate change' is happening when in the real world it isn't. Imperial College is driving the climate agenda as it drives the 'Covid' agenda (both Cult hoaxes) while Patrick Vallance, the UK government's Chief Scientific Adviser on 'Covid', was named Chief Scientific Adviser to the UN 'climate change' conference known as COP26 hosted by the government in Glasgow, Scotland. 'Covid' and 'climate' are fundamentally connected.

Professor Woeful

From Imperial's bosom came Neil Ferguson still advising government despite his previous disasters and it was announced early on that he and other key people like UK Chief Medical Adviser Chris Whitty had caught the 'virus' as the propaganda story was being sold. Somehow they managed to survive and we had Prime Minister Boris Johnson admitted to hospital with what was said to be a severe version of the 'virus' in this same period. His whole policy and demeanour changed when he returned to Downing Street. It's a small world with these government advisors – especially in their communal connections to Gates – and Ferguson had partnered with Whitty to write a paper called 'Infectious disease: Tough choices to reduce Ebola transmission' which involved another scare-story that didn't happen. Ferguson's 'models' predicted that up to150, 000 could die from 'mad cow disease', or BSE, and its version in sheep if it was transmitted to humans. BSE was not transmitted and instead triggered by an organophosphate pesticide used to treat a pest on

cows. Fewer than 200 deaths followed from the human form. Models by Ferguson and his fellow incompetents led to the unnecessary culling of millions of pigs, cattle and sheep in the foot and mouth outbreak in 2001 which destroyed the lives and livelihoods of farmers and their families who had often spent decades building their herds and flocks. Vast numbers of these animals did not have foot and mouth and had no contact with the infection. Another 'expert' behind the cull was Professor Roy Anderson, a computer modeller at Imperial College specialising in the epidemiology of *human*, not animal, disease. Anderson has served on the Bill and Melinda Gates Grand Challenges in Global Health advisory board and chairs another Gates-funded organisation. Gates is everywhere.

In a precursor to the 'Covid' script Ferguson backed closing schools 'for prolonged periods' over the swine flu 'pandemic' in 2009 and said it would affect a third of the world population if it continued to spread at the speed he claimed to be happening. His mates at Imperial College said much the same and a news report said: 'One of the authors, the epidemiologist and disease modeller Neil Ferguson, who sits on the World Health Organisation's emergency committee for the outbreak, said the virus had "full pandemic potential".' Professor Liam Donaldson, the Chris Whitty of his day as Chief Medical Officer, said the worst case could see 30 percent of the British people infected by swine flu with 65,000 dying. Ferguson and Donaldson were indeed proved correct when at the end of the year the number of deaths attributed to swine flu was 392. The term 'expert' is rather liberally applied unfortunately, not least to complete idiots. Swine flu 'projections' were great for GlaxoSmithKline (GSK) as millions rolled in for its Pandemrix influenza vaccine which led to brain damage with children most affected. The British government (taxpayers) paid out more than £60 million in compensation after GSK was given immunity from prosecution. Yet another 'Covid' déjà vu. Swine flu was supposed to have broken out in Mexico, but Dr Wolfgang Wodarg, a German doctor, former member of parliament and critic of the 'Covid' hoax, observed 'the spread of swine flu' in Mexico City at the time. He

said: 'What we experienced in Mexico City was a very mild flu which did not kill more than usual – which killed even fewer people than usual.' Hyping the fear against all the facts is not unique to 'Covid' and has happened many times before. Ferguson is reported to have over-estimated the projected death toll of bird flu (H5N1) by some three million-fold, but bird flu vaccine makers again made a killing from the scare. This is some of the background to the Neil Ferguson who produced the perfectly-timed computer models in early 2020 predicting that half a million people would die in Britain without draconian lockdown and 2.2 million in the United States. Politicians panicked, people panicked, and lockdowns of alleged short duration were instigated to 'flatten the curve' of cases gleaned from a test not testing for the 'virus'. I said at the time that the public could forget the 'short duration' bit. This was an agenda to destroy the livelihoods of the population and force them into mass control through dependency and there was going to be nothing 'short' about it. American researcher Daniel Horowitz described the consequences of the 'models' spewed out by Gates-funded Ferguson and Imperial College:

> What led our government and the governments of many other countries into panic was a single Imperial College of UK study, funded by global warming activists, that predicted 2.2 million deaths if we didn't lock down the country. In addition, the reported 8-9% death rate in Italy scared us into thinking there was some other mutation of this virus that they got, which might have come here.
>
> Together with the fact that we were finally testing and had the ability to actually report new cases, we thought we were headed for a death spiral. But again … we can't flatten a curve if we don't know when the curve started.

How about it *never* started?

Giving them what they want

An investigation by German news outlet *Welt Am Sonntag (World on Sunday)* revealed how in March, 2020, the German government gathered together 'leading scientists from several research institutes and universities' and 'together, they were to produce a [modelling]

paper that would serve as legitimization for further tough political measures'. The Cult agenda was justified by computer modelling not based on evidence or reality; it was specifically constructed to justify the Cult demand for lockdowns all over the world to destroy the independent livelihoods of the global population. All these modellers and everyone responsible for the 'Covid' hoax have a date with a trial like those in Nuremberg after World War Two when Nazis faced the consequences of their war crimes. These corrupt-beyond-belief 'modellers' wrote the paper according to government instructions and it said that that if lockdown measures were lifted then up to one million Germans would die from 'Covid-19' adding that some would die 'agonizingly at home, gasping for breath' unable to be treated by hospitals that couldn't cope. All lies. No matter – it gave the Cult all that it wanted. What did long-time government 'modeller' Neil Ferguson say? If the UK and the United States didn't lockdown half a million would die in Britain and 2.2 million Americans. Anyone see a theme here? 'Modellers' are such a crucial part of the lockdown strategy that we should look into their background and follow the money. Researcher Rosemary Frei produced an excellent article headlined 'The Modelling-paper Mafiosi'. She highlights a guy called John Edmunds, a British epidemiologist, and professor in the Faculty of Epidemiology and Population Health at the London School of Hygiene & Tropical Medicine. He studied at Imperial College. Edmunds is a member of government 'Covid' advisory bodies which have been dictating policy, the New and Emerging Respiratory Virus Threats Advisory Group (NERVTAG) and the Scientific Advisory Group for Emergencies (SAGE).

Ferguson, another member of NERVTAG and SAGE, led the way with the original 'virus' and Edmunds has followed in the 'variant' stage and especially the so-called UK or Kent variant known as the 'Variant of Concern' (VOC) B.1.1.7. He said in a co-written report for the Centre for Mathematical modelling of Infectious Diseases at the London School of Hygiene and Tropical Medicine, with input from the Centre's 'Covid-19' Working Group, that there was 'a realistic

possibility that VOC B.1.1.7 is associated with an increased risk of death compared to non-VOC viruses'. Fear, fear, fear, get the vaccine, fear, fear, fear, get the vaccine. Rosemary Frei reveals that almost all the paper's authors and members of the modelling centre's 'Covid-19' Working Group receive funding from the Bill and Melinda Gates Foundation and/or the associated Gates-funded Wellcome Trust. The paper was published by e-journal *Medr χiv* which only publishes papers not peer-reviewed and the journal was established by an organisation headed by Facebook's Mark Zuckerberg and his missus. What a small world it is. Frei discovered that Edmunds is on the Scientific Advisory Board of the Coalition for Epidemic Preparedness Innovations (CEPI) which was established by the Bill and Melinda Gates Foundation, Klaus Schwab's Davos World Economic Forum and Big Pharma giant Wellcome. CEPI was 'launched in Davos [in 2017] to develop vaccines to stop future epidemics', according to its website. 'Our mission is to accelerate the development of vaccines against emerging infectious diseases and enable equitable access to these vaccines for people during outbreaks.' What kind people they are. Rosemary Frei reveals that Public Health England (PHE) director Susan Hopkins is an author of her organisation's non-peer-reviewed reports on 'new variants'. Hopkins is a professor of infectious diseases at London's Imperial College which is gifted tens of millions of dollars a year by the Bill and Melinda Gates Foundation. Gates-funded modelling disaster Neil Ferguson also co-authors Public Health England reports and he spoke in December, 2020, about the potential danger of the B.1.1.7. 'UK variant' promoted by Gates-funded modeller John Edmunds. When I come to the 'Covid vaccines' the 'new variants' will be shown for what they are – bollocks.

Connections, connections

All these people and modellers are lockdown-obsessed or, put another way, they demand what the Cult demands. Edmunds said in January, 2021, that to ease lockdowns too soon would be a disaster and they had to 'vaccinate much, much, much more widely than the

elderly'. Rosemary Frei highlights that Edmunds is married to Jeanne Pimenta who is described in a LinkedIn profile as director of epidemiology at GlaxoSmithKline (GSK) and she held shares in the company. Patrick Vallance, co-chair of SAGE and the government's Chief Scientific Adviser, is a former executive of GSK and has a deferred bonus of shares in the company worth £600,000. GSK has serious business connections with Bill Gates and is collaborating with mRNA-'vaccine' company CureVac to make 'vaccines' for the new variants that Edmunds is talking about. GSK is planning a 'Covid vaccine' with drug giant Sanofi. Puppet Prime Minister Boris Johnson announced in the spring of 2021 that up to 60 million vaccine doses were to be made at the GSK facility at Barnard Castle in the English North East. Barnard Castle, with a population of just 6,000, was famously visited in breach of lockdown rules in April, 2020, by Johnson aide Dominic Cummings who said that he drove there 'to test his eyesight' before driving back to London. Cummings would be better advised to test his integrity – not that it would take long. The GSK facility had nothing to do with his visit then although I'm sure Patrick Vallance would have been happy to arrange an introduction and some tea and biscuits. Ruthless psychopath Gates has made yet another fortune from vaccines in collaboration with Big Pharma companies and gushes at the phenomenal profits to be made from vaccines – more than a 20-to-1 return as he told one interviewer. Gates also tweeted in December, 2019, with the foreknowledge of what was coming: 'What's next for our foundation? I'm particularly excited about what the next year could mean for one of the best buys in global health: vaccines.'

Modeller John Edmunds is a big promotor of vaccines as all these people appear to be. He's the dean of the London School of Hygiene & Tropical Medicine's Faculty of Epidemiology and Population Health which is primarily funded by the Bill and Melinda Gates Foundation and the Gates-established and funded GAVI vaccine alliance which is the Gates vehicle to vaccinate the world. The organisation Doctors Without Borders has described GAVI as being 'aimed more at supporting drug-industry desires to promote new

products than at finding the most efficient and sustainable means for fighting the diseases of poverty'. But then that's why the psychopath Gates created it. John Edmunds said in a video that the London School of Hygiene & Tropical Medicine is involved in every aspect of vaccine development including large-scale clinical trials. He contends that mathematical modelling can show that vaccines protect individuals and society. That's on the basis of shit in and shit out, I take it. Edmunds serves on the UK Vaccine Network as does Ferguson and the government's foremost 'Covid' adviser, the grim-faced, dark-eyed Chris Whitty. The Vaccine Network says it works 'to support the government to identify and shortlist targeted investment opportunities for the most promising vaccines and vaccine technologies that will help combat infectious diseases with epidemic potential, and to address structural issues related to the UK's broader vaccine infrastructure'. Ferguson is acting Director of the Imperial College Vaccine Impact Modelling Consortium which has funding from the Bill and Melina Gates Foundation and the Gates-created GAVI 'vaccine alliance'. Anyone wonder why these characters see vaccines as the answer to every problem? Ferguson is wildly enthusiastic in his support for GAVI's campaign to vaccine children en masse in poor countries. You would expect someone like Gates who has constantly talked about the need to reduce the population to want to fund vaccines to keep more people alive. I'm sure that's why he does it. The John Edmunds London School of Hygiene & Tropical Medicine (LSHTM) has a Vaccines Manufacturing Innovation Centre which develops, tests and commercialises vaccines. Rosemary Frei writes:

> The vaccines centre also performs affiliated activities like combating 'vaccine hesitancy'. The latter includes the Vaccine Confidence Project. The project's stated purpose is, among other things, 'to provide analysis and guidance for early response and engagement with the public to ensure sustained confidence in vaccines and immunisation'. The Vaccine Confidence Project's director is LSHTM professor Heidi Larson. For more than a decade she's been researching how to combat vaccine hesitancy.

How the bloody hell can blokes like John Edmunds and Neil Ferguson with those connections and financial ties model 'virus' case

and death projections for the government and especially in a way that gives their paymasters like Gates exactly what they want? It's insane, but this is what you find throughout the world.

'Covid' is not dangerous, oops, wait, yes it is

Only days before Ferguson's nightmare scenario made Jackboot Johnson take Britain into a China-style lockdown to save us from a deadly 'virus' the UK government website gov.uk was reporting something very different to Ferguson on a page of official government guidance for 'high consequence infectious diseases (HCID)'. It said this about 'Covid-19':

As of 19 March 2020, COVID-19 *is no longer considered to be a high consequence infectious diseases (HCID) in the UK* [my emphasis]. The 4 nations public health HCID group made an interim recommendation in January 2020 to classify COVID-19 as an HCID. This was based on consideration of the UK HCID criteria about the virus and the disease with information available during the early stages of the outbreak.

Now that more is known about COVID-19, the public health bodies in the UK have reviewed the most up to date information about COVID-19 against the UK HCID criteria. They have determined that several features have now changed; in particular, more information is available about mortality rates (low overall), and there is now greater clinical awareness and a specific and sensitive laboratory test, the availability of which continues to increase. The Advisory Committee on Dangerous Pathogens (ACDP) is also of the opinion that COVID-19 should no longer be classified as an HCID.

Soon after the government had been exposed for downgrading the risk they upgraded it again and everyone was back to singing from the same Cult hymn book. Ferguson and his fellow Gates clones indicated that lockdowns and restrictions would have to continue until a Gates-funded vaccine was developed. Gates said the same because Ferguson and his like were repeating the Gates script which is the Cult script. 'Flatten the curve' became an ongoing nightmare of continuing lockdowns with periods in between of severe restrictions in pursuit of destroying independent incomes and had nothing to do with protecting health about which the Cult gives not a shit. Why wouldn't Ferguson be pushing a vaccine 'solution' when he's owned by vaccine-obsessive Gates who makes a fortune from them and

when Ferguson heads the Vaccine Impact Modelling Consortium at Imperial College funded by the Gates Foundation and GAVI, the 'vaccine alliance', created by Gates as his personal vaccine promotion operation? To compound the human catastrophe that Ferguson's 'models' did so much to create he was later exposed for breaking his own lockdown rules by having sexual liaisons with his married girlfriend Antonia Staats at his home while she was living at another location with her husband and children. Staats was a 'climate' activist and senior campaigner at the Soros-funded Avaaz which I wouldn't trust to tell me that grass is green. Ferguson had to resign as a government advisor over this hypocrisy in May, 2020, but after a period of quiet he was back being quoted by the ridiculous media on the need for more lockdowns and a vaccine rollout. Other government-advising 'scientists' from Imperial College' held the fort in his absence and said lockdown could be indefinite until a vaccine was found. The Cult script was being sung by the payrolled choir. I said there was no intention of going back to 'normal' when the 'vaccine' came because the 'vaccine' is part of a very different agenda that I will discuss in Human 2.0. Why would the Cult want to let the world go back to normal when destroying that normal forever was the whole point of what was happening? House arrest, closing businesses and schools through lockdown, (un)social distancing and masks all followed the Ferguson fantasy models. Again as I predicted (these people are so predictable) when the 'vaccine' arrived we were told that house arrest, lockdown, (un)social distancing and masks would still have to continue. I will deal with the masks in the next chapter because they are of fundamental importance.

Where's the 'pandemic'?

Any mildly in-depth assessment of the figures revealed what was really going on. Cult-funded and controlled organisations still have genuine people working within them such is the number involved. So it is with Genevieve Briand, assistant program director of the Applied Economics master's degree program at Johns Hopkins

University. She analysed the impact that 'Covid-19' had on deaths from *all* causes in the United States using official data from the CDC for the period from early February to early September, 2020. She found that allegedly 'Covid' *related*-deaths exceeded those from heart disease which she found strange with heart disease always the biggest cause of fatalities. Her research became even more significant when she noted the sudden decline in 2020 of *all* non-'Covid' deaths: 'This trend is completely contrary to the pattern observed in all previous years … the total decrease in deaths by other causes almost exactly equals the increase in deaths by Covid-19.' This was such a game, set and match in terms of what was happening that Johns Hopkins University deleted the article on the grounds that it 'was being used to support false and dangerous inaccuracies about the impact of the pandemic'. No – because it exposed the scam from official CDC figures and this was confirmed when those figures were published in January, 2021. Here we can see the effect of people dying from heart attacks, cancer, road accidents and gunshot wounds – *anything* – having 'Covid-19' on the death certificate along with those diagnosed from 'symptoms' who had even not tested positive with a test not testing for the 'virus'. I am not kidding with the gunshot wounds, by the way. Brenda Bock, coroner in Grand County, Colorado, revealed that two gunshot victims tested positive for the 'virus' within the previous 30 days and were therefore classified as 'Covid deaths'. Bock said: 'These two people had tested positive for Covid, but that's not what killed them. A gunshot wound is what killed them.' She said she had not even finished her investigation when the state listed the gunshot victims as deaths due to the 'virus'. The death and case figures for 'Covid-19' are an absolute joke and yet they are repeated like parrots by the media, politicians and alleged medical 'experts'. The official Cult narrative is the only show in town.

Genevieve Briand found that deaths from all causes were not exceptional in 2020 compared with previous years and a Spanish magazine published figures that said the same about Spain which was a 'Covid' propaganda hotspot at one point. *Discovery Salud*, a

health and medicine magazine, quoted government figures which showed how 17,000 *fewer* people died in Spain in 2020 than in 2019 and more than 26,000 fewer than in 2018. The age-standardised mortality rate for England and Wales when age distribution is taken into account was significantly lower in 2020 than the 1970s, 80s and 90s, and was only the ninth highest since 2000. Where is the 'pandemic'?

Post mortems and autopsies virtually disappeared for 'Covid' deaths amid claims that 'virus-infected' bodily fluids posed a risk to those carrying out the autopsy. This was rejected by renowned German pathologist and forensic doctor Klaus Püschel who said that he and his staff had by then done 150 autopsies on 'Covid' patients with no problems at all. He said they were needed to know why some 'Covid' patients suffered blood clots and not severe respiratory infections. The 'virus' is, after all, called SARS or 'severe acute respiratory syndrome'. I highlighted in the spring of 2020 this phenomenon and quoted New York intensive care doctor Cameron Kyle-Sidell who posted a soon deleted YouTube video to say that they had been told to prepare to treat an infectious disease called 'Covid-19', but that was not what they were dealing with. Instead he likened the lung condition of the most severely ill patients to what you would expect with cabin depressurisation in a plane at 30,000 feet or someone dropped on the top of Everest without oxygen or acclimatisation. I have never said this is not happening to a small minority of alleged 'Covid' patients – I am saying this is not caused by a phantom 'contagious virus'. Indeed Kyle-Sidell said that 'Covid-19' was not the disease they were told was coming their way. 'We are operating under a medical paradigm that is untrue,' he said, and he believed they were treating the wrong disease: 'These people are being slowly starved of oxygen.' Patients would take off their oxygen masks in a state of fear and stress and while they were blue in the face on the brink of death. They did not look like patients dying of pneumonia. You can see why they don't want autopsies when their virus doesn't exist and there is another condition in some people that they don't wish to be uncovered. I should add here that

the 5G system of millimetre waves was being rapidly introduced around the world in 2020 and even more so now as they fire 5G at the Earth from satellites. At 60 gigahertz within the 5G range that frequency interacts with the oxygen molecule and stops people breathing in sufficient oxygen to be absorbed into the bloodstream. They are installing 5G in schools and hospitals. The world is not mad or anything. 5G can cause major changes to the lungs and blood as I detail in *The Answer* and these consequences are labelled 'Covid-19', the alleged symptoms of which can be caused by 5G and other electromagnetic frequencies as cells respond to radiation poisoning.

The 'Covid death' scam

Dr Scott Jensen, a Minnesota state senator and medical doctor, exposed 'Covid' Medicare payment incentives to hospitals and death certificate manipulation. He said he was sent a seven-page document by the US Department of Health 'coaching' him on how to fill out death certificates which had never happened before. The document said that he didn't need to have a laboratory test for 'Covid-19' to put that on the death certificate and that shocked him when death certificates are supposed to be about facts. Jensen described how doctors had been 'encouraged, if not pressured' to make a diagnosis of 'Covid-19' if they thought it was probable or *'presumed*'. No positive test was necessary – not that this would have mattered anyway. He said doctors were told to diagnose 'Covid' by symptoms when these were the same as colds, allergies, other respiratory problems, and certainly with influenza which 'disappeared' in the 'Covid' era. A common sniffle was enough to get the dreaded verdict. Ontario authorities decreed that a single care home resident with *one* symptom from a long list must lead to the isolation of the entire home. Other courageous doctors like Jensen made the same point about death figure manipulation and how deaths by other causes were falling while 'Covid-19 deaths' were rising at the same rate due to re-diagnosis. Their videos rarely survive long on YouTube with its Cult-supporting algorithms courtesy of CEO Susan Wojcicki and her bosses at Google. Figure-tampering was so glaring

and ubiquitous that even officials were letting it slip or outright saying it. UK chief scientific adviser Patrick Vallance said on one occasion that 'Covid' on the death certificate doesn't mean 'Covid' was the cause of death (so why the hell is it there?) and we had the rare sight of a BBC reporter telling the truth when she said: 'Someone could be successfully treated for Covid, in say April, discharged, and then in June, get run over by a bus and die … That person would still be counted as a Covid death in England.' Yet the BBC and the rest of the world media went on repeating the case and death figures as if they were real. Illinois Public Health Director Dr Ngozi Ezike revealed the deceit while her bosses must have been clenching their buttocks:

> If you were in a hospice and given a few weeks to live and you were then found to have Covid that would be counted as a Covid death. [There might be] a clear alternate cause, but it is still listed as a Covid death. So everyone listed as a Covid death doesn't mean that was the cause of the death, but that they had Covid at the time of death.

Yes, a 'Covid virus' never shown to exist and tested for with a test not testing for the 'virus'. In the first period of the pandemic hoax through the spring of 2020 the process began of designating almost everything a 'Covid' death and this has continued ever since. I sat in a restaurant one night listening to a loud conversation on the next table where a family was discussing in bewilderment how a relative who had no symptoms of 'Covid', and had died of a long-term problem, could have been diagnosed a death by the 'virus'. I could understand their bewilderment. If they read this book they will know why this medical fraud has been perpetrated the world over.

Some media truth shock

The media ignored the evidence of death certificate fraud until eventually one columnist did speak out when she saw it first-hand. Bel Mooney is a long-time national newspaper journalist in Britain currently working for the *Daily Mail*. Her article on February 19th, 2021, carried this headline: 'My dad Ted passed three Covid tests

and died of a chronic illness yet he's officially one of Britain's 120,000 victims of the virus and is far from alone ... so how many more are there?' She told how her 99-year-old father was in a care home with a long-standing chronic obstructive pulmonary disease and vascular dementia. Maybe, but he was still aware enough to tell her from the start that there was no 'virus' and he refused the 'vaccine' for that reason. His death was not unexpected given his chronic health problems and Mooney said she was shocked to find that 'Covid-19' was declared the cause of death on his death certificate. She said this was a 'bizarre and unacceptable untruth' for a man with long-time health problems who had tested negative twice at the home for the 'virus'. I was also shocked by this story although not by what she said. I had been highlighting the death certificate manipulation for ten months. It was the confirmation that a professional full-time journalist only realised this was going on when it affected her directly and neither did she know that whether her dad tested positive or negative was irrelevant with the test not testing for the 'virus'. Where had she been? She said she did not believe in 'conspiracy theories' without knowing I'm sure that this and 'conspiracy theorists' were terms put into widespread circulation by the CIA in the 1960s to discredit those who did not accept the ridiculous official story of the Kennedy assassination. A blanket statement of 'I don't believe in conspiracy theories' is always bizarre. The dictionary definition of the term alone means the world is drowning in conspiracies. What she said was even more daft when her dad had just been affected by the 'Covid' conspiracy. Why else does she think that 'Covid-19' was going on the death certificates of people who died of something else?

To be fair once she saw from personal experience what was happening she didn't mince words. Mooney was called by the care home on the morning of February 9th to be told her father had died in his sleep. When she asked for the official cause of death what came back was 'Covid-19'. Mooney challenged this and was told there had been deaths from Covid on the dementia floor (confirmed by a test not testing for the 'virus') so they considered it 'reasonable

to assume'. 'But doctor,' Mooney rightly protested, 'an assumption isn't a diagnosis.' She said she didn't blame the perfectly decent and sympathetic doctor – 'he was just doing his job'. Sorry, but that's *bullshit*. He wasn't doing his job at all. He was putting a false cause of death on the death certificate and that is a criminal offence for which he should be brought to account and the same with the millions of doctors worldwide who have done the same. They were not doing their job they were following orders and that must not wash at new Nuremberg trials any more than it did at the first ones. Mooney's doctor was 'assuming' (presuming) as he was told to, but 'just following orders' makes no difference to his actions. A doctor's job is to serve the patient and the truth, not follow orders, but that's what they have done all over the world and played a central part in making the 'Covid' hoax possible with all its catastrophic consequences for humanity. Shame on them and they must answer for their actions. Mooney said her disquiet worsened when she registered her father's death by telephone and was told by the registrar there had been very many other cases like hers where 'the deceased' had not tested positive for 'Covid' yet it was recorded as the cause of death. The test may not matter, but those involved at their level *think* it matters and it shows a callous disregard for accurate diagnosis. The pressure to do this is coming from the top of the national 'health' pyramids which in turn obey the World Health Organization which obeys Gates and the Cult. Mooney said the registrar agreed that this must distort the national figures adding that 'the strangest thing is that every winter we record countless deaths from flu, and this winter there have been none. Not one!' She asked if the registrar thought deaths from flu were being misdiagnosed and lumped together with 'Covid' deaths. The answer was a 'puzzled yes'. Mooney said that the funeral director said the same about 'Covid' deaths which had nothing to do with 'Covid'. They had lost count of the number of families upset by this and other funeral companies in different countries have had the same experience. Mooney wrote:

The nightly shroud-waving and shocking close-ups of pain imposed on us by the TV news bewildered and terrified the population into eager compliance with lockdowns. We were invited to 'save the NHS' and to grieve for strangers – the real-life loved ones behind those shocking death counts. Why would the public imagine what I now fear, namely that the way Covid-19 death statistics are compiled might make the numbers seem greater than they are?

Oh, just a little bit – like 100 percent.

Do the maths

Mooney asked why a country would wish to skew its mortality figures by wrongly certifying deaths? What had been going on? Well, if you don't believe in conspiracies you will never find the answer which is that *it's a conspiracy*. She did, however, describe what she had discovered as a 'national scandal'. In reality it's a global scandal and happening everywhere. Pillars of this conspiracy were all put into place before the button was pressed with the Drosten PCR protocol and high amplifications to produce the cases and death certificate changes to secure illusory 'Covid' deaths. Mooney notes that normally two doctors were needed to certify a death, with one having to know the patient, and how the rules were changed in the spring of 2020 to allow one doctor to do this. In the same period 'Covid deaths' were decreed to be all cases where Covid-19 was put on the death certificate even without a positive test or any symptoms. Mooney asked: 'How many of the 30,851 (as of January 15) care home resident deaths with Covid-19 on the certificate (32.4 per cent of all deaths so far) were based on an assumption, like that of my father? And what has that done to our national psyche?'All of them is the answer to the first question and it has devastated and dismantled the national psyche, actually the global psyche, on a colossal scale. In the UK case and death data is compiled by organisations like Public Health England (PHE) and the Office for National Statistics (ONS). Mooney highlights the insane policy of counting a death from any cause as 'Covid-19' if this happens within 28 days of a positive test (with a test not testing for the 'virus') and she points out that ONS statistics reflect deaths 'involving Covid' 'or due to Covid' which meant in practice any

death where 'Covid-19' was mentioned on the death certificate. She described the consequences of this fraud:

Most people will accept the narrative they are fed, so panicky governments here and in Europe witnessed the harsh measures enacted in totalitarian China and jumped into lockdown. Headlines about Covid deaths tolled like the knell that would bring doomsday to us all. Fear stalked our empty streets. Politicians parroted the frankly ridiculous aim of 'zero Covid' and shut down the economy, while most British people agreed that lockdown was essential and (astonishingly to me, as a patriotic Brit) even wanted more restrictions.

For what? Lies on death certificates? Never mind the grim toll of lives ruined, suicides, schools closed, rising inequality, depression, cancelled hospital treatments, cancer patients in a torture of waiting, poverty, economic devastation, loneliness, families kept apart, and so on. How many lives have been lost as a direct result of lockdown?

She said that we could join in a national chorus of shock and horror at reaching the 120,000 death toll which was surely certain to have been totally skewed all along, but what about the human cost of lockdown justified by these 'death figures'? *The British Medical Journal* had reported a 1,493 percent increase in cases of children taken to Great Ormond Street Hospital with abusive head injuries alone and then there was the effect on families:

Perhaps the most shocking thing about all this is that families have been kept apart – and obeyed the most irrational, changing rules at the whim of government – because they believed in the statistics. They succumbed to fear, which his generation rejected in that war fought for freedom. Dad (God rest his soul) would be angry. And so am I.

Another theme to watch is that in the winter months when there are more deaths from all causes they focus on 'Covid' deaths and in the summer when the British Lung Foundation says respiratory disease plummets by 80 percent they rage on about 'cases'. Either way fascism on population is always the answer.

Nazi eugenics in the 21st century

Elderly people in care homes have been isolated from their families month after lonely month with no contact with relatives and grandchildren who were banned from seeing them. We were told

that lockdown fascism was to 'protect the vulnerable' like elderly people. At the same time Do Not Resuscitate (DNR) orders were placed on their medical files so that if they needed resuscitation it wasn't done and 'Covid-19' went on their death certificates. Old people were not being 'protected' they were being culled – murdered in truth. DNR orders were being decreed for disabled and young people with learning difficulties or psychological problems. The UK Care Quality Commission, a non-departmental body of the Department of Health and Social Care, found that 34 percent of those working in health and social care were pressured into placing 'do not attempt cardiopulmonary resuscitation' orders on 'Covid' patients who suffered from disabilities and learning difficulties without involving the patient or their families in the decision. UK judges ruled that an elderly woman with dementia should have the DNA-manipulating 'Covid vaccine' against her son's wishes and that a man with severe learning difficulties should have the jab despite his family's objections. Never mind that many had already died. The judiciary always supports doctors and government in fascist dictatorships. They wouldn't dare do otherwise. A horrific video was posted showing fascist officers from Los Angeles police forcibly giving the 'Covid' shot to women with special needs who were screaming that they didn't want it. The same fascists are seen giving the jab to a sleeping elderly woman in a care home. This is straight out of the Nazi playbook. Hitler's Nazis committed mass murder of the mentally ill and physically disabled throughout Germany and occupied territories in the programme that became known as Aktion T4, or just T4. Sabbatian-controlled Hitler and his grotesque crazies set out to kill those they considered useless and unnecessary. The Reich Committee for the Scientific Registering of Hereditary and Congenital Illnesses registered the births of babies identified by physicians to have 'defects'. By 1941 alone more than 5,000 children were murdered by the state and it is estimated that in total the number of innocent people killed in Aktion T4 was between 275,000 and 300,000. Parents were told their children had been sent away for 'special treatment' never to return. It is rather pathetic to see claims about plans for new extermination camps being dismissed today

when the same force behind current events did precisely that 80 years ago. Margaret Sanger was a Cult operative who used 'birth control' to sanitise her programme of eugenics. Organisations she founded became what is now Planned Parenthood. Sanger proposed that 'the whole dysgenic population would have its choice of segregation or sterilization'. These included epileptics, 'feeble-minded', and prostitutes. Sanger opposed charity because it perpetuated 'human waste'. She reveals the Cult mentality and if anyone thinks that extermination camps are a 'conspiracy theory' their naivety is touching if breathtakingly stupid.

If you don't believe that doctors can act with callous disregard for their patients it is worth considering that doctors and medical staff agreed to put government-decreed DNR orders on medical files and do nothing when resuscitation is called for. I don't know what you call such people in your house. In mine they are Nazis from the Josef Mengele School of Medicine. Phenomenal numbers of old people have died worldwide from the effects of lockdown, depression, lack of treatment, the 'vaccine' (more later) and losing the will to live. A common response at the start of the manufactured pandemic was to remove old people from hospital beds and transfer them to nursing homes. The decision would result in a mass cull of elderly people in those homes through lack of treatment – *not* 'Covid'. Care home whistleblowers have told how once the 'Covid' era began doctors would not come to their homes to treat patients and they were begging for drugs like antibiotics that often never came. The most infamous example was ordered by New York governor Andrew Cuomo, brother of a moronic CNN host, who amazingly was given an Emmy Award for his handling of the 'Covid crisis' by the ridiculous Wokers that hand them out. Just how ridiculous could be seen in February, 2021, when a Department of Justice and FBI investigation began into how thousands of old people in New York died in nursing homes after being discharged from hospital to make way for 'Covid' patients on Cuomo's say-so – and how he and his staff covered up these facts. This couldn't have happened to a nicer psychopath. Even then there was a 'Covid' spin. Reports said that

thousands of old people who tested positive for 'Covid' in hospital were transferred to nursing homes to both die of 'Covid' and transmit it to others. No – they were in hospital because they were ill and the fact that they tested positive with a test not testing for the 'virus' is irrelevant. They were ill often with respiratory diseases ubiquitous in old people near the end of their lives. Their transfer out of hospital meant that their treatment stopped and many would go on to die.

They're old. Who gives a damn?

I have exposed in the books for decades the Cult plan to cull the world's old people and even to introduce at some point what they call a 'demise pill' which at a certain age everyone would take and be out of here by law. In March, 2021, Spain legalised euthanasia and assisted suicide following the Netherlands, Belgium, Luxembourg and Canada on the Tiptoe to the demise pill. Treatment of old people by many 'care' homes has been a disgrace in the 'Covid' era. There are many, many, caring staff – I know some. There have, however, been legions of stories about callous treatment of old people and their families. Police were called when families came to take their loved ones home in the light of isolation that was killing them. They became prisoners of the state. Care home residents in insane, fascist Ontario, Canada, were not allowed to leave their *room* once the 'Covid' hoax began. UK staff have even wheeled elderly people away from windows where family members were talking with them. Oriana Criscuolo from Stockport in the English North West dropped off some things for her 80-year-old father who has Parkinson's disease and dementia and she wanted to wave to him through a ground-floor window. She was told that was 'illegal'. When she went anyway they closed the curtains in the middle of the day. Oriana said:

> It's just unbelievable. I cannot understand how care home staff – people who are being paid to care – have become so uncaring. Their behaviour is inhumane and cruel. It's beyond belief.

She was right and this was not a one-off. What a way to end your life in such loveless circumstances. UK registered nurse Nicky Millen, a proper old school nurse for 40 years, said that when she started her career care was based on dignity, choice, compassion and empathy. Now she said 'the things that are important to me have gone out of the window.' She was appalled that people were dying without their loved ones and saying goodbye on iPads. Nicky described how a distressed 89-year-old lady stroked her face and asked her 'how many paracetamol would it take to finish me off'. Life was no longer worth living while not seeing her family. Nicky said she was humiliated in front of the ward staff and patients for letting the lady stroke her face and giving her a cuddle. Such is the dehumanisation that the 'Covid' hoax has brought to the surface. Nicky worked in care homes where patients told her they were being held prisoner. 'I want to live until I die', one said to her. 'I had a lady in tears because she hadn't seen her great-grandson.' Nicky was compassionate old school meeting psychopathic New Normal. She also said she had worked on a 'Covid' ward with no 'Covid' patients. Jewish writer Shai Held wrote an article in March, 2020, which was headlined 'The Staggering, Heartless Cruelty Toward the Elderly'. What he described was happening from the earliest days of lockdown. He said 'the elderly' were considered a group and not unique individuals (the way of the Woke). Shai Held said:

Notice how the all-too-familiar rhetoric of dehumanization works: 'The elderly' are bunched together as a faceless mass, all of them considered culprits and thus effectively deserving of the suffering the pandemic will inflict upon them. Lost entirely is the fact that the elderly are individual human beings, each with a distinctive face and voice, each with hopes and dreams, memories and regrets, friendships and marriages, loves lost and loves sustained.

'The elderly' have become another dehumanised group for which anything goes and for many that has resulted in cold disregard for their rights and their life. The distinctive face that Held talks about is designed to be deleted by masks until everyone is part of a faceless mass.

‘War-zone’ hospitals myth

Again and again medical professionals have told me what was really going on and how hospitals ‘overrun like war zones’ according to the media were virtually empty. The mantra from medical whistleblowers was please don’t use my name or my career is over. Citizen journalists around the world sneaked into hospitals to film evidence exposing the ‘war-zone’ lie. They really *were* largely empty with closed wards and operating theatres. I met a hospital worker in my town on the Isle of Wight during the first lockdown in 2020 who said the only island hospital had never been so quiet. Lockdown was justified by the psychopaths to stop hospitals being overrun. At the same time that the island hospital was near-empty the military arrived here to provide *extra beds*. It was all propaganda to ramp up the fear to ensure compliance with fascism as were never-used temporary hospitals with thousands of beds known as Nightingales and never-used make-shift mortuaries opened by the criminal UK government. A man who helped to install those extra island beds attributed to the army said they were never used and the hospital was empty. Doctors and nurses ‘stood around talking or on their phones, wandering down to us to see what we were doing’. There were no masks or social distancing. He accused the useless local island paper, the *County Press*, of ‘pumping the fear as if our hospital was overrun and we only have one so it should have been’. He described ambulances parked up with crews outside in deck chairs. When his brother called an ambulance he was told there was a two-hour backlog which he called ‘bullshit’. An old lady on the island fell ‘and was in a bad way’, but a caller who rang for an ambulance was told the situation wasn’t urgent enough. Ambulance stations were working under capacity while people would hear ambulances with sirens blaring driving through the streets. When those living near the stations realised what was going on they would follow them as they left, circulated around an urban area with the sirens going, and then came back without stopping. All this was to increase levels of fear and the same goes for the ‘ventilator shortage crisis’ that cost tens of millions for hastily produced ventilators never to be used.

Ambulance crews that agreed to be exploited in this way for fear propaganda might find themselves a mirror. I wish them well with that. Empty hospitals were the obvious consequence of treatment and diagnoses of non-'Covid' conditions cancelled and those involved handed a death sentence. People have been dying at home from undiagnosed and untreated cancer, heart disease and other life-threatening conditions to allow empty hospitals to deal with a 'pandemic' that wasn't happening.

Death of the innocent

'War-zones' have been laying off nursing staff, even doctors where they can. There was no work for them. Lockdown was justified by saving lives and protecting the vulnerable they were actually killing with DNR orders and preventing empty hospitals being 'overrun'. In Britain the mantra of stay at home to 'save the NHS' was everywhere and across the world the same story was being sold when it was all lies. Two California doctors, Dan Erickson and Artin Massihi at Accelerated Urgent Care in Bakersfield, held a news conference in April, 2020, to say that intensive care units in California were 'empty, essentially', with hospitals shutting floors, not treating patients and laying off doctors. The California health system was working at minimum capacity 'getting rid of doctors because we just don't have the volume'. They said that people with conditions such as heart disease and cancer were not coming to hospital out of fear of 'Covid-19'. Their video was deleted by Susan Wojcicki's Cult-owned YouTube after reaching five million views. Florida governor Ron Desantis, who rejected the severe lockdowns of other states and is being targeted for doing so, said that in March, 2020, every US governor was given models claiming they would run out of hospital beds in days. That was never going to happen and the 'modellers' knew it. Deceit can be found at every level of the system. Urgent children's operations were cancelled including fracture repairs and biopsies to spot cancer. Eric Nicholls, a consultant paediatrician, said 'this is obviously concerning and we need to return to normal operating and to increase capacity as soon as possible'. Psychopaths

in power were rather less concerned *because* they are psychopaths. Deletion of urgent care and diagnosis has been happening all over the world and how many kids and others have died as a result of the actions of these cold and heartless lunatics dictating 'health' policy? The number must be stratospheric. Richard Sullivan, professor of cancer and global health at King's College London, said people feared 'Covid' more than cancer such was the campaign of fear. 'Years of lost life will be quite dramatic', Sullivan said, with 'a huge amount of avoidable mortality'. Sarah Woolnough, executive director for policy at Cancer Research UK, said there had been a 75 percent drop in urgent referrals to hospitals by family doctors of people with suspected cancer. Sullivan said that 'a lot of services have had to scale back – we've seen a dramatic decrease in the amount of elective cancer surgery'. Lockdown deaths worldwide has been absolutely fantastic with the *New York Post* reporting how data confirmed that 'lockdowns end more lives than they save':

> There was a sharp decline in visits to emergency rooms and an increase in fatal heart attacks because patients didn't receive prompt treatment. Many fewer people were screened for cancer. Social isolation contributed to excess deaths from dementia and Alzheimer's.
>
> Researchers predicted that the social and economic upheaval would lead to tens of thousands of "deaths of despair" from drug overdoses, alcoholism and suicide. As unemployment surged and mental-health and substance-abuse treatment programs were interrupted, the reported levels of anxiety, depression and suicidal thoughts increased dramatically, as did alcohol sales and fatal drug overdoses.

This has been happening while nurses and other staff had so much time on their hands in the 'war-zones' that Tic-Tok dancing videos began appearing across the Internet with medical staff dancing around in empty wards and corridors as people died at home from causes that would normally have been treated in hospital.

Mentions in dispatches

One brave and truth-committed whistleblower was Louise Hampton, a call handler with the UK NHS who made a viral Internet video saying she had done 'fuck all' during the 'pandemic'

which was 'a load of bollocks'. She said that 'Covid-19' was rebranded flu and of course she lost her job. This is what happens in the medical and endless other professions now when you tell the truth. Louise filmed inside 'war-zone' accident and emergency departments to show they were empty and I mean *empty* as in no one there. The mainstream media could have done the same and blown the gaff on the whole conspiracy. They haven't to their eternal shame. Not that most 'journalists' seem capable of manifesting shame as with the psychopaths they slavishly repeat without question. The relative few who were admitted with serious health problems were left to die alone with no loved ones allowed to see them because of 'Covid' rules and they included kids dying without the comfort of mum and dad at their bedside while the evil behind this couldn't give a damn. It was all good fun to them. A Scottish NHS staff nurse publicly quit in the spring of 2021 saying: 'I can no longer be part of the lies and the corruption by the government.' She said hospitals 'aren't full, the beds aren't full, beds have been shut, wards have been shut'. Hospitals were never busy throughout 'Covid'. The staff nurse said that Nicola Sturgeon, tragically the leader of the Scottish government, was on television saying save the hospitals and the NHS – 'but the beds are empty' and 'we've not seen flu, we always see flu every year'. She wrote to government and spoke with her union Unison (the unions are Cult-compromised and *useless*, but nothing changed. Many of her colleagues were scared of losing their jobs if they spoke out as they wanted to. She said nursing staff were being affected by wearing masks all day and 'my head is splitting every shift from wearing a mask'. The NHS is part of the fascist tyranny and must be dismantled so we can start again with human beings in charge. (Ironically, hospitals were reported to be busier again when official 'Covid' cases *fell* in spring/summer of 2021 and many other conditions required treatment at the same time as *the fake vaccine rollout*.)

I will cover the 'Covid vaccine' scam in detail later, but it is another indicator of the sickening disregard for human life that I am highlighting here. The DNA-manipulating concoctions do not fulfil

the definition of a 'vaccine', have never been used on humans before and were given only emergency approval because trials were not completed and they continued using the unknowing public. The result was what a NHS senior nurse with responsibility for 'vaccine' procedure said was 'genocide'. She said the 'vaccines' were not 'vaccines'. They had not been shown to be safe and claims about their effectiveness by drug companies were 'poetic licence'. She described what was happening as a 'horrid act of human annihilation'. The nurse said that management had instigated a policy of not providing a Patient Information Leaflet (PIL) before people were 'vaccinated' even though health care professionals are supposed to do this according to protocol. Patients should also be told that they are taking part in an ongoing clinical trial. Her challenges to what is happening had seen her excluded from meetings and ridiculed in others. She said she was told to 'watch my step … or I would find myself surplus to requirements'. The nurse, who spoke anonymously in fear of her career, said she asked her NHS manager why he/she was content with taking part in genocide against those having the 'vaccines'. The reply was that everyone had to play their part and to 'put up, shut up, and get it done'. Government was 'leaning heavily' on NHS management which was clearly leaning heavily on staff. This is how the global 'medical' hierarchy operates and it starts with the Cult and its World Health Organization.

She told the story of a doctor who had the Pfizer jab and when questioned had no idea what was in it. The doctor had never read the literature. We have to stop treating doctors as intellectual giants when so many are moral and medical pygmies. The doctor did not even know that the 'vaccines' were not fully approved or that their trials were ongoing. They were, however, asking their patients if they minded taking part in follow-ups for research purposes – yes, the *ongoing clinical trial*. The nurse said the doctor's ignorance was not rare and she had spoken to a hospital consultant who had the jab without any idea of the background or that the 'trials' had not been completed. Nurses and pharmacists had shown the same ignorance.

'My NHS colleagues have forsaken their duty of care, broken their code of conduct – Hippocratic Oath – and have been brainwashed just the same as the majority of the UK public through propaganda …' She said she had not been able to recruit a single NHS colleague, doctor, nurse or pharmacist to stand with her and speak out. Her union had refused to help. She said that if the genocide came to light she would not hesitate to give evidence at a Nuremberg-type trial against those in power who could have affected the outcomes but didn't.

And all for what?

To put the nonsense into perspective let's say the 'virus' does exist and let's go completely crazy and accept that the official manipulated figures for cases and deaths are accurate. *Even then* a study by Stanford University epidemiologist Dr John Ioannidis published on the World Health Organization website produced an average infection to fatality rate of … *0.23 percent!* Ioannidis said: 'If one could sample equally from all locations globally, the median infection fatality rate might even be substantially lower than the 0.23% observed in my analysis.' For healthy people under 70 it was … *0.05 percent*! This compares with the 3.4 percent claimed by the Cult-owned World Health Organization when the hoax was first played and maximum fear needed to be generated. An updated Stanford study in April, 2021, put the 'infection' to 'fatality' rate at just 0.15 percent. Another team of scientists led by Megan O'Driscoll and Henrik Salje studied data from 45 countries and published their findings on the Nature website. For children and young people the figure is so small it virtually does not register although authorities will be hyping dangers to the young when they introduce DNA-manipulating 'vaccines' for children. The O'Driscoll study produced an average infection-fatality figure of 0.003 for children from birth to four; 0.001 for 5 to 14; 0.003 for 15 to 19; and it was still only 0.456 up to 64. To claim that children must be 'vaccinated' to protect them from 'Covid' is an obvious lie and so there must be another reason and there is. What's more the average age of a 'Covid' death is akin

to the average age that people die in general. The average age of death in England is about 80 for men and 83 for women. The average age of death from alleged 'Covid' is between 82 and 83. California doctors, Dan Erickson and Artin Massihi, said at their April media conference that projection models of millions of deaths had been 'woefully inaccurate'. They produced detailed figures showing that Californians had a 0.03 chance of dying from 'Covid' based on the number of people who tested positive (with a test not testing for the 'virus'). Erickson said there was a 0.1 percent chance of dying from 'Covid' in the *state* of New York, not just the city, and a 0.05 percent chance in Spain, a centre of 'Covid-19' hysteria at one stage. The Stanford studies supported the doctors' data with fatality rate estimates of 0.23 and 0.15 percent. How close are these figures to my estimate of *zero*? Death-rate figures claimed by the World Health Organization at the start of the hoax were some 15 times higher. The California doctors said there was no justification for lockdowns and the economic devastation they caused. Everything they had ever learned about quarantine was that you quarantine the *sick* and not the healthy. They had never seen this before and it made no medical sense.

Why in the in the light of all this would governments and medical systems the world over say that billions must go under house arrest; lose their livelihood; in many cases lose their mind, their health and their life; force people to wear masks dangerous to health and psychology; make human interaction and even family interaction a criminal offence; ban travel; close restaurants, bars, watching live sport, concerts, theatre, and any activity involving human togetherness and discourse; and closing schools to isolate children from their friends and cause many to commit suicide in acts of hopelessness and despair? The California doctors said lockdown consequences included increased child abuse, partner abuse, alcoholism, depression, and other impacts they were seeing every day. Who would do that to the entire human race if not mentally-ill psychopaths of almost unimaginable extremes like Bill Gates? We must face the reality of what we are dealing with and come out of

denial. Fascism and tyranny are made possible only by the target population submitting and acquiescing to fascism and tyranny. The whole of human history shows that to be true. Most people naively and unquestioning believed what they were told about a 'deadly virus' and meekly and weakly submitted to house arrest. Those who didn't believe it – at least in total – still submitted in fear of the consequences of not doing so. For the rest who wouldn't submit draconian fines have been imposed, brutal policing by psychopaths *for* psychopaths, and condemnation from the meek and weak who condemn the Pushbackers on behalf of the very force that has them, too, in its gunsights. 'Pathetic' does not even begin to suffice. Britain's brainless 'Health' Secretary Matt Hancock warned anyone lying to border officials about returning from a list of 'hotspot' countries could face a jail sentence of up to ten years which is more than for racially-aggravated assault, incest and attempting to have sex with a child under 13. Hancock is a lunatic, but he has the state apparatus behind him in a Cult-led chain reaction and the same with UK 'Vaccine Minister' Nadhim Zahawi, a prominent member of the mega-Cult secret society, Le Cercle, which featured in my earlier books. The Cult enforces its will on governments and medical systems; government and medical systems enforce their will on business and police; business enforces its will on staff who enforce it on customers; police enforce the will of the Cult on the population and play their essential part in creating a world of fascist control that their own children and grandchildren will have to live in their entire lives. It is a hierarchical pyramid of imposition and acquiescence and, yes indeedy, of clinical insanity.

Does anyone bright enough to read this book have to ask what the answer is? I think not, but I will reveal it anyway in the fewest of syllables: Tell the psychos and their moronic lackeys to fuck off and let's get on with our lives. We are many – They are few.

CHAPTER SEVEN

War on your mind

One believes things because one has been conditioned to believe them
Aldous Huxley, *Brave New World*

I have described the 'Covid' hoax as a 'Psyop' and that is true in every sense and on every level in accordance with the definition of that term which is psychological warfare. Break down the 'Covid pandemic' to the foundation themes and it is psychological warfare on the human individual and collective mind.

The same can be said for the entire human belief system involving every subject you can imagine. Huxley was right in his contention that people believe what they are conditioned to believe and this comes from the repetition throughout their lives of the same falsehoods. They spew from government, corporations, media and endless streams of 'experts' telling you what the Cult wants you to believe and often believing it themselves (although *far* from always). 'Experts' are rewarded with 'prestigious' jobs and titles and as agents of perceptual programming with regular access to the media. The Cult has to control the narrative – control *information* – or they lose control of the vital, crucial, without-which-they-cannot-prevail public perception of reality. The foundation of that control today is the Internet made possible by the Defense Advanced Research Projects Agency (DARPA), the incredibly sinister technological arm of the Pentagon. The Internet is the result of military technology.

DARPA openly brags about establishing the Internet which has been a long-term project to lasso the minds of the global population. I have said for decades the plan is to control information to such an extreme that eventually no one would see or hear anything that the Cult does not approve. We are closing in on that end with ferocious censorship since the 'Covid' hoax began and in my case it started back in the 1990s in terms of books and speaking venues. I had to create my own publishing company in 1995 precisely because no one else would publish my books even then. I think they're all still running.

Cult Internet

To secure total control of information they needed the Internet in which pre-programmed algorithms can seek out 'unclean' content for deletion and even stop it being posted in the first place. The Cult had to dismantle print and non-Internet broadcast media to ensure the transfer of information to the appropriate-named 'Web' – a critical expression of the *Cult* web. We've seen the ever-quickening demise of traditional media and control of what is left by a tiny number of corporations operating worldwide. Independent journalism in the mainstream is already dead and never was that more obvious than since the turn of 2020. The Cult wants all information communicated via the Internet to globally censor and allow the plug to be pulled any time. Lockdowns and forced isolation has meant that communication between people has been through electronic means and no longer through face-to-face discourse and discussion. Cult psychopaths have targeted the bars, restaurants, sport, venues and meeting places in general for this reason. None of this is by chance and it's to stop people gathering in any kind of privacy or number while being able to track and monitor all Internet communications and block them as necessary. Even private messages between individuals have been censored by these fascists that control Cult fronts like Facebook, Twitter, Google and YouTube which are all officially run by Sabbatian place-people and from the background by higher-level Sabbatian place people.

Facebook, Google, Amazon and their like were seed-funded and supported into existence with money-no-object infusions of funds either directly or indirectly from DARPA and CIA technology arm In-Q-Tel. The Cult plays the long game and prepares very carefully for big plays like 'Covid'. Amazon is another front in the psychological war and pretty much controls the global market in book sales and increasingly publishing. Amazon's limitless funds have deleted fantastic numbers of independent publishers to seize global domination on the way to deciding which books can be sold and circulated and which cannot. Moves in that direction are already happening. Amazon's leading light Jeff Bezos is the grandson of Lawrence Preston Gise who worked with DARPA predecessor ARPA. Amazon has big connections to the CIA and the Pentagon. The plan I have long described went like this:

1. Employ military technology to establish the Internet.

2. Sell the Internet as a place where people can freely communicate without censorship and allow that to happen until the Net becomes the central and irreversible pillar of human society. If the Internet had been highly censored from the start many would have rejected it.

3. Fund and manipulate major corporations into being to control the circulation of information on your Internet using cover stories about geeks in garages to explain how they came about. Give them unlimited funds to expand rapidly with no need to make a profit for years while non-Cult companies who need to balance the books cannot compete. You know that in these circumstances your Googles, YouTubes, Facebooks and Amazons are going to secure near monopolies by either crushing or buying up the opposition.

4. Allow freedom of expression on both the Internet and communication platforms to draw people in until the Internet is the central and irreversible pillar of human society and your communication corporations have reached a stage of near monopoly domination.

5. Then unleash your always-planned frenzy of censorship on the basis of 'where else are you going to go?' and continue to expand that until nothing remains that the Cult does not want its human targets to see.

The process was timed to hit the 'Covid' hoax to ensure the best chance possible of controlling the narrative which they knew they had to do at all costs. They were, after all, about to unleash a 'deadly virus' that didn't really exist. If you do that in an environment of free-flowing information and opinion you would be dead in the

water before you could say Gates is a psychopath. The network was in place through which the Cult-created-and-owned World Health Organization could dictate the 'Covid' narrative and response policy slavishly supported by Cult-owned Internet communication giants and mainstream media while those telling a different story were censored. Google, YouTube, Facebook and Twitter openly announced that they would do this. What else would we expect from Cult-owned operations like Facebook which former executives have confirmed set out to make the platform more addictive than cigarettes and coldly manipulates emotions of its users to sow division between people and groups and scramble the minds of the young? If Zuckerberg lives out the rest of his life without going to jail for crimes against humanity, and most emphatically against the young, it will be a travesty of justice. Still, no matter, cause and effect will catch up with him eventually and the same with Sergey Brin and Larry Page at Google with its CEO Sundar Pichai who fix the Google search results to promote Cult narratives and hide the opposition. Put the same key words into Google and other search engines like DuckDuckGo and you will see how different results can be. Wikipedia is another intensely biased 'encyclopaedia' which skews its content to the Cult agenda. YouTube links to Wikipedia's version of 'Covid' and 'climate change' on video pages in which experts in their field offer a different opinion (even that is increasingly rare with Wojcicki censorship). Into this 'Covid' silence-them network must be added government media censors, sorry 'regulators', such as Ofcom in the UK which imposed tyrannical restrictions on British broadcasters that had the effect of banning me from ever appearing. Just to debate with me about my evidence and views on 'Covid' would mean breaking the fascistic impositions of Ofcom and its CEO career government bureaucrat Melanie Dawes. Gutless British broadcasters tremble at the very thought of fascist Ofcom.

Psychos behind 'Covid'

The reason for the 'Covid' catastrophe in all its facets and forms can be seen by whom and what is driving the policies worldwide in such a coordinated way. Decisions are not being made to protect health, but to target psychology. The dominant group guiding and 'advising' government policy are not medical professionals. They are psychologists and behavioural scientists. Every major country has its own version of this phenomenon and I'll use the British example to show how it works. In many ways the British version has been affecting the wider world in the form of the huge behaviour manipulation network in the UK which operates in other countries. The network involves private companies, government, intelligence and military. The Cabinet Office is at the centre of the government 'Covid' Psyop and part-owns, with 'innovation charity' Nesta, the Behavioural Insights Team (BIT) which claims to be independent of government but patently isn't. The BIT was established in 2010 and its job is to manipulate the psyche of the population to acquiesce to government demands and so much more. It is also known as the 'Nudge Unit', a name inspired by the 2009 book by two ultra-Zionists, Cass Sunstein and Richard Thaler, called *Nudge: Improving Decisions About Health, Wealth, and Happiness*. The book, as with the Behavioural Insights Team, seeks to 'nudge' behaviour (manipulate it) to make the public follow patterns of action and perception that suit those in authority (the Cult). Sunstein is so skilled at this that he advises the World Health Organization and the UK Behavioural Insights Team and was Administrator of the White House Office of Information and Regulatory Affairs in the Obama administration. Biden appointed him to the Department of Homeland Security – another ultra-Zionist in the fold to oversee new immigration laws which is another policy the Cult wants to control. Sunstein is desperate to silence anyone exposing conspiracies and co-authored a 2008 report on the subject in which suggestions were offered to ban 'conspiracy theorizing' or impose 'some kind of tax, financial or otherwise, on those who disseminate such theories'. I guess a psychiatrist's chair is out of the question?

Sunstein's mate Richard Thaler, an 'academic affiliate' of the UK Behavioural Insights Team, is a proponent of 'behavioural economics' which is defined as the study of 'the effects of psychological, cognitive, emotional, cultural and social factors on the decisions of individuals and institutions'. Study the effects so they can be manipulated to be what you want them to be. Other leading names in the development of behavioural economics are ultra-Zionists Daniel Kahneman and Robert J. Shiller and they, with Thaler, won the Nobel Memorial Prize in Economic Sciences for their work in this field. The Behavioural Insights Team is operating at the heart of the UK government and has expanded globally through partnerships with several universities including Harvard, Oxford, Cambridge, University College London (UCL) and Pennsylvania. They claim to have 'trained' (reframed) 20,000 civil servants and run more than 750 projects involving 400 randomised controlled trials in dozens of countries' as another version of mind reframers Common Purpose. BIT works from its office in New York with cities and their agencies, as well as other partners, across the United States and Canada – this is a company part-owned by the British government Cabinet Office. An executive order by President Cult-servant Obama established a US Social and Behavioral Sciences Team in 2015. They all have the same reason for being and that's to brainwash the population directly and by brainwashing those in positions of authority.

'Covid' mind game

Another prime aspect of the UK mind-control network is the 'independent' [joke] Scientific Pandemic Insights Group on Behaviours (SPI-B) which 'provides behavioural science advice aimed at anticipating and helping people adhere to interventions that are recommended by medical or epidemiological experts'. That means manipulating public perception and behaviour to do whatever government tells them to do. It's disgusting and if they really want the public to be 'safe' this lot should all be under lock and key. According to the government website SPI-B consists of

'behavioural scientists, health and social psychologists, anthropologists and historians' and advises the Whitty-Vallance-led Scientific Advisory Group for Emergencies (SAGE) which in turn advises the government on 'the science' (it doesn't) and 'Covid' policy. When politicians say they are being guided by 'the science' this is the rabble in each country they are talking about and that 'science' is dominated by behaviour manipulators to enforce government fascism through public compliance. The Behaviour Insight Team is headed by psychologist David Solomon Halpern, a visiting professor at King's College London, and connects with a national and global web of other civilian and military organisations as the Cult moves towards its goal of fusing them into one fascistic whole in every country through its 'Fusion Doctrine'. The behaviour manipulation network involves, but is not confined to, the Foreign Office; National Security Council; government communications headquarters (GCHQ); MI5; MI6; the Cabinet Office-based Media Monitoring Unit; and the Rapid Response Unit which 'monitors digital trends to spot emerging issues; including misinformation and disinformation; and identifies the best way to respond'.

There is also the 77th Brigade of the UK military which operates like the notorious Israeli military's Unit 8200 in manipulating information and discussion on the Internet by posing as members of the public to promote the narrative and discredit those who challenge it. Here we have the military seeking to manipulate *domestic* public opinion while the Nazis in government are fine with that. Conservative Member of Parliament Tobias Ellwood, an advocate of lockdown and control through 'vaccine passports', is a Lieutenant Colonel reservist in the 77th Brigade which connects with the military operation jHub, the 'innovation centre' for the Ministry of Defence and Strategic Command. jHub has also been involved with the civilian National Health Service (NHS) in 'symptom tracing' the population. The NHS is a key part of this mind control network and produced a document in December, 2020, explaining to staff how to use psychological manipulation with different groups and ages to get them to have the DNA-manipulating 'Covid vaccine'

that's designed to cumulatively rewrite human genetics. The document, called 'Optimising Vaccination Roll Out – Do's and Dont's for all messaging, documents and "communications" in the widest sense', was published by NHS England and the NHS Improvement *Behaviour Change Unit* in partnership with Public Health England and Warwick Business School. I hear the mantra about 'save the NHS' and 'protect the NHS' when we need to scrap the NHS and start again. The current version is far too corrupt, far too anti-human and totally compromised by Cult operatives and their assets. UK government broadcast media censor Ofcom will connect into this web – as will the BBC with its tremendous Ofcom influence – to control what the public see and hear and dictate mass perception. Nuremberg trials must include personnel from all these organisations.

The fear factor

The 'Covid' hoax has led to the creation of the UK Cabinet Office-connected Joint Biosecurity Centre (JBC) which is officially described as providing 'expert advice on pandemics' using its independent [all Cult operations are 'independent'] analytical function to provide real-time analysis about infection outbreaks to identify and respond to outbreaks of Covid-19'. Another role is to advise the government on a response to spikes in infections – 'for example by closing schools or workplaces in local areas where infection levels have risen'. Put another way, promoting the Cult agenda. The Joint Biosecurity Centre is modelled on the Joint Terrorism Analysis Centre which analyses intelligence to set 'terrorism threat levels' and here again you see the fusion of civilian and military operations and intelligence that has led to military intelligence producing documents about 'vaccine hesitancy' and how it can be combated. Domestic civilian matters and opinions should not be the business of the military. The Joint Biosecurity Centre is headed by Tom Hurd, director general of the Office for Security and Counter-Terrorism from the establishment-to-its-fingertips Hurd family. His father is former Foreign Secretary Douglas Hurd. How coincidental that Tom

Hurd went to the elite Eton College and Oxford University with Boris Johnson. Imperial College with its ridiculous computer modeller Neil Ferguson will connect with this gigantic web that will itself interconnect with similar set-ups in other major and not so major countries. Compared with this Cult network the politicians, be they Boris Johnson, Donald Trump or Joe Biden, are bit-part players 'following the science'. The network of psychologists was on the 'Covid' case from the start with the aim of generating maximum fear of the 'virus' to ensure compliance by the population. A government behavioural science group known as SPI-B produced a paper in March, 2020, for discussion by the main government science advisory group known as SAGE. It was headed 'Options for increasing adherence to social distancing measures' and it said the following in a section headed 'Persuasion':

- A substantial number of people still do not feel sufficiently personally threatened; it could be that they are reassured by the low death rate in their demographic group, although levels of concern may be rising. Having a good understanding of the risk has been found to be positively associated with adoption of COVID-19 social distancing measures in Hong Kong.

- The perceived level of personal threat needs to be increased among those who are complacent, using hard-hitting evaluation of options for increasing social distancing emotional messaging. To be effective this must also empower people by making clear the actions they can take to reduce the threat.

- Responsibility to others: There seems to be insufficient understanding of, or feelings of responsibility about, people's role in transmitting the infection to others … Messaging about actions need to be framed positively in terms of protecting oneself and the community, and increase confidence that they will be effective.

- Some people will be more persuaded by appeals to play by the rules, some by duty to the community, and some to personal risk.

> All these different approaches are needed. The messaging also needs to take account of the realities of different people's lives. Messaging needs to take account of the different motivational levers and circumstances of different people.

All this could be achieved the SPI-B psychologists said by *using the media to increase the sense of personal threat* which translates as terrify the shit out of the population, including children, so they all do what we want. That's not happened has it? Those excuses for 'journalists' who wouldn't know journalism if it bit them on the arse (the great majority) have played their crucial part in serving this Cult-government Psyop to enslave their own kids and grandkids. How they live with themselves I have no idea. The psychological war has been underpinned by constant government 'Covid' propaganda in almost every television and radio ad break, plus the Internet and print media, which has pounded out the fear with taxpayers footing the bill for their own programming. The result has been people terrified of a 'virus' that doesn't exist or one with a tiny fatality rate even if you believe it does. People walk down the street and around the shops wearing face-nappies damaging their health and psychology while others report those who refuse to be that naïve to the police who turn up in their own face-nappies. I had a cameraman come to my flat and he was so frightened of 'Covid' he came in wearing a mask and refused to shake my hand in case he caught something. He had – naïveitis – and the thought that he worked in the mainstream media was both depressing and made his behaviour perfectly explainable. The fear which has gripped the minds of so many and frozen them into compliance has been carefully cultivated by these psychologists who are really psychopaths. If lives get destroyed and a lot of young people commit suicide it shows our plan is working. SPI-B then turned to compulsion on the public to comply. 'With adequate preparation, rapid change can be achieved', it said. Some countries had introduced mandatory self-isolation on a wide scale without evidence of major public unrest and a large majority of the UK's population appeared to be supportive of more coercive measures with 64 percent of adults saying they would

support putting London under a lockdown (watch the 'polls' which are designed to make people believe that public opinion is in favour or against whatever the subject in hand).

For 'aggressive protective measures' to be effective, the SPI-B paper said, special attention should be devoted to those population groups that are more at risk. Translated from the Orwellian this means making the rest of population feel guilty for not protecting the 'vulnerable' such as old people which the Cult and its agencies were about to kill on an industrial scale with lockdown, lack of treatment and the Gates 'vaccine'. Psychopath psychologists sold their guilt-trip so comprehensively that Los Angeles County Supervisor Hilda Solis reported that children were apologising (from a distance) to their parents and grandparents for bringing 'Covid' into their homes and getting them sick. '… These apologies are just some of the last words that loved ones will ever hear as they die alone,' she said. Gut-wrenchingly Solis then used this childhood tragedy to tell children to stay at home and 'keep your loved ones alive'. Imagine heaping such potentially life-long guilt on a kid when it has absolutely nothing to do with them. These people are deeply disturbed and the psychologists behind this even more so.

Uncivil war – divide and rule

Professional mind-controllers at SPI-B wanted the media to increase a sense of responsibility to others (do as you're told) and promote 'positive messaging' for those actions while in contrast to invoke 'social disapproval' by the unquestioning, obedient, community of anyone with a mind of their own. Again the compliant Goebbels-like media obliged. This is an old, old, trick employed by tyrannies the world over throughout human history. You get the target population to keep the target population in line – *your* line. SPI-B said this could 'play an important role in preventing anti-social behaviour or discouraging failure to enact pro-social behaviour'. For 'anti-social' in the Orwellian parlance of SPI-B see any behaviour that government doesn't approve. SPI-B recommendations said that 'social disapproval' should be accompanied by clear messaging and

promotion of strong collective identity – hence the government and celebrity mantra of 'we're all in this together'. Sure we are. The mind doctors have such contempt for their targets that they think some clueless comedian, actor or singer telling them to do what the government wants will be enough to win them over. We have had UK comedian Lenny Henry, actor Michael Caine and singer Elton John wheeled out to serve the propagandists by urging people to have the DNA-manipulating 'Covid' non-'vaccine'. The role of Henry and fellow black celebrities in seeking to coax a 'vaccine' reluctant black community into doing the government's will was especially stomach-turning. An emotion-manipulating script and carefully edited video featuring these black 'celebs' was such an insult to the intelligence of black people and where's the self-respect of those involved selling their souls to a fascist government agenda? Henry said he heard black people's 'legitimate worries and concerns', but people must 'trust the facts' when they were doing exactly that by not having the 'vaccine'. They had to include the obligatory reference to Black Lives Matter with the line … 'Don't let coronavirus cost even more black lives – because we matter'. My god, it was pathetic. 'I know the vaccine is safe and what it does.' How? 'I'm a comedian and it says so in my script.'

SPI-B said social disapproval needed to be carefully managed to avoid victimisation, scapegoating and misdirected criticism, but they knew that their 'recommendations' would lead to exactly that and the media were specifically used to stir-up the divide-and-conquer hostility. Those who conform like good little baa, baas, are praised while those who have seen through the tidal wave of lies are 'Covidiots'. The awake have been abused by the fast asleep for not conforming to fascism and impositions that the awake know are designed to endanger their health, dehumanise them, and tear asunder the very fabric of human society. We have had the curtain-twitchers and morons reporting neighbours and others to the face-nappied police for breaking 'Covid rules' with fascist police delighting in posting links and phone numbers where this could be done. The Cult cannot impose its will without a compliant police

and military or a compliant population willing to play their part in enslaving themselves and their kids. The words of a pastor in Nazi Germany are so appropriate today:

First they came for the socialists and I did not speak out because I was not a socialist.

Then they came for the trade unionists and I did not speak out because I was not a trade unionist.

Then they came for the Jews and I did not speak out because I was not a Jew.

Then they came for me and there was no one left to speak for me.

Those who don't learn from history are destined to repeat it and so many are.

'Covid' rules: Rewiring the mind

With the background laid out to this gigantic national and global web of psychological manipulation we can put 'Covid' rules into a clear and sinister perspective. Forget the claims about protecting health. 'Covid' rules are about dismantling the human mind, breaking the human spirit, destroying self-respect, and then putting Humpty Dumpty together again as a servile, submissive slave. Social isolation through lockdown and distancing have devastating effects on the human psyche as the psychological psychopaths well know and that's the real reason for them. Humans need contact with each other, discourse, closeness and touch, or they eventually, and literarily, go crazy. Masks, which I will address at some length, fundamentally add to the effects of isolation and the Cult agenda to dehumanise and de-individualise the population. To do this while knowing – in fact *seeking* – this outcome is the very epitome of evil and psychologists involved in this *are* the epitome of evil. They must like all the rest of the Cult demons and their assets stand trial for crimes against humanity on a scale that defies the imagination. Psychopaths in uniform use isolation to break enemy troops and agents and make them subservient and submissive to tell what they know. The technique is rightly considered a form of torture and

torture is most certainly what has been imposed on the human population.

Clinically-insane American psychologist Harry Harlow became famous for his isolation experiments in the 1950s in which he separated baby monkeys from their mothers and imprisoned them for months on end in a metal container or 'pit of despair'. They soon began to show mental distress and depression as any idiot could have predicted. Harlow put other monkeys in steel chambers for three, six or twelve months while denying them any contact with animals or humans. He said that the effects of total social isolation for six months were 'so devastating and debilitating that we had assumed initially that twelve months of isolation would not produce any additional decrement'; but twelve months of isolation 'almost obliterated the animals socially'. This is what the Cult and its psychopaths are doing to you and your children. Even monkeys in partial isolation in which they were not allowed to form relationships with other monkeys became 'aggressive and hostile, not only to others, but also towards their own bodies'. We have seen this in the young as a consequence of lockdown. UK government psychopaths launched a public relations campaign telling people not to hug each other even after they received the 'Covid-19 vaccine' which we were told with more lies would allow a return to 'normal life'. A government source told *The Telegraph*: 'It will be along the lines that it is great that you have been vaccinated, but if you are going to visit your family and hug your grandchildren there is a chance you are going to infect people you love.' The source was apparently speaking from a secure psychiatric facility. Janet Lord, director of Birmingham University's Institute of Inflammation and Ageing, said that parents and grandparents should avoid hugging their children. Well, how can I put it, Ms Lord? Fuck off. Yep, that'll do.

Destroying the kids – where are the parents?

Observe what has happened to people enslaved and isolated by lockdown as suicide and self-harm has soared worldwide,

particularly among the young denied the freedom to associate with their friends. A study of 49,000 people in English-speaking countries concluded that almost half of young adults are at clinical risk of mental health disorders. A national survey in America of 1,000 currently enrolled high school and college students found that 5 percent reported attempting suicide during the pandemic. Data from the US CDC's National Syndromic Surveillance Program from January 1st to October 17th, 2020, revealed a *31 percent* increase in mental health issues among adolescents aged 12 to 17 compared with 2019. The CDC reported that America in general suffered the biggest drop in life expectancy since World War Two as it fell by a year in the first half of 2020 as a result of 'deaths of despair' – overdoses and suicides. Deaths of despair have leapt by more than 20 percent during lockdown and include the highest number of fatal overdoses ever recorded in a single year – 81,000. Internet addiction is another consequence of being isolated at home which lowers interest in physical activities as kids fall into inertia and what's the point? Children and young people are losing hope and giving up on life, sometimes literally. A 14-year-old boy killed himself in Maryland because he had 'given up' when his school district didn't reopen; an 11-year-old boy shot himself during a zoom class; a teenager in Maine succumbed to the isolation of the 'pandemic' when he ended his life after experiencing a disrupted senior year at school. Children as young as nine have taken their life and all these stories can be repeated around the world. Careers are being destroyed before they start and that includes those in sport in which promising youngsters have not been able to take part. The plan of the psycho-psychologists is working all right. Researchers at Cambridge University found that lockdowns cause significant harm to children's mental health. Their study was published in the *Archives of Disease in Childhood*, and followed 168 children aged between 7 and 11. The researchers concluded:

> During the UK lockdown, children's depression symptoms have increased substantially, relative to before lockdown. The scale of this effect has direct relevance for the continuation of different elements of lockdown policy, such as complete or partial school closures …

… Specifically, we observed a statistically significant increase in ratings of depression, with a medium-to-large effect size. Our findings emphasise the need to incorporate the potential impact of lockdown on child mental health in planning the ongoing response to the global pandemic and the recovery from it.

Not a chance when the Cult's psycho-psychologists were getting exactly what they wanted. The UK's Royal College of Paediatrics and Child Health has urged parents to look for signs of eating disorders in children and young people after a three to four fold increase. Specialists say the 'pandemic' is a major reason behind the rise. You don't say. The College said isolation from friends during school closures, exam cancellations, loss of extra-curricular activities like sport, and an increased use of social media were all contributory factors along with fears about the virus (psycho-psychologists again), family finances, and students being forced to quarantine. Doctors said young people were becoming severely ill by the time they were seen with 'Covid' regulations reducing face-to-face consultations. Nor is it only the young that have been devastated by the psychopaths. Like all bullies and cowards the Cult is targeting the young, elderly, weak and infirm. A typical story was told by a British lady called Lynn Parker who was not allowed to visit her husband in 2020 for the last ten and half months of his life 'when he needed me most' between March 20th and when he died on December 19th. This vacates the criminal and enters the territory of evil. The emotional impact on the immune system alone is immense as are the number of people of all ages worldwide who have died as a result of Cult-demanded, Gates-demanded, lockdowns.

Isolation is torture

The experience of imposing solitary confinement on millions of prisoners around the world has shown how a large percentage become 'actively psychotic and/or acutely suicidal'. Social isolation has been found to trigger 'a specific psychiatric syndrome, characterized by hallucinations; panic attacks; overt paranoia; diminished impulse control; hypersensitivity to external stimuli; and difficulties with thinking, concentration and memory'. Juan Mendez,

a United Nations rapporteur (investigator), said that isolation is a form of torture. Research has shown that even after isolation prisoners find it far more difficult to make social connections and I remember chatting to a shop assistant after one lockdown who told me that when her young son met another child again he had no idea how to act or what to do. Hannah Flanagan, Director of Emergency Services at Journey Mental Health Center in Dane County, Wisconsin, said: 'The specificity about Covid social distancing and isolation that we've come across as contributing factors to the suicides are really new to us this year.' But they are not new to those that devised them. They are getting the effect they want as the population is psychologically dismantled to be rebuilt in a totally different way. Children and the young are particularly targeted. They will be the adults when the full-on fascist AI-controlled technocracy is planned to be imposed and they are being prepared to meekly submit. At the same time older people who still have a memory of what life was like before – and how fascist the new normal really is – are being deleted. You are going to see efforts to turn the young against the old to support this geriatric genocide. Hannah Flanagan said the big increase in suicide in her county proved that social isolation is not only harmful, but deadly. Studies have shown that isolation from others is one of the main risk factors in suicide and even more so with women. Warnings that lockdown could create a 'perfect storm' for suicide were ignored. After all this was one of the *reasons* for lockdown. Suicide, however, is only the most extreme of isolation consequences. There are many others. Dr Dhruv Khullar, assistant professor of healthcare policy at Weill Cornell Medical College, said in a *New York Times* article in 2016 long before the fake 'pandemic':

> A wave of new research suggests social separation is bad for us. Individuals with less social connection have disrupted sleep patterns, altered immune systems, more inflammation and higher levels of stress hormones. One recent study found that isolation increases the risk of heart disease by 29 percent and stroke by 32 percent. Another analysis that pooled data from 70 studies and 3.4 million people found that socially isolated individuals had a 30 percent higher risk of dying in the next seven years, and that this effect was largest in middle age.

Loneliness can accelerate cognitive decline in older adults, and isolated individuals are twice as likely to die prematurely as those with more robust social interactions. These effects start early: Socially isolated children have significantly poorer health 20 years later, even after controlling for other factors. All told, loneliness is as important a risk factor for early death as obesity and smoking.

There you have proof from that one article alone four years before 2020 that those who have enforced lockdown, social distancing and isolation knew what the effect would be and that is even more so with professional psychologists that have been driving the policy across the globe. We can go back even further to the years 2000 and 2003 and the start of a major study on the effects of isolation on health by Dr Janine Gronewold and Professor Dirk M. Hermann at the University Hospital in Essen, Germany, who analysed data on 4,316 people with an average age of 59 who were recruited for the long-term research project. They found that socially isolated people are more than 40 percent more likely to have a heart attack, stroke, or other major cardiovascular event and nearly 50 percent more likely to die from any cause. Given the financial Armageddon unleashed by lockdown we should note that the study found a relationship between increased cardiovascular risk and lack of financial support. After excluding other factors social isolation was still connected to a 44 percent increased risk of cardiovascular problems and a 47 percent increased risk of death by any cause. Lack of financial support was associated with a 30 percent increase in the risk of cardiovascular health events. Dr Gronewold said it had been known for some time that feeling lonely or lacking contact with close friends and family can have an impact on physical health and the study had shown that having strong social relationships is of high importance for heart health. Gronewold said they didn't understand yet why people who are socially isolated have such poor health outcomes, but this was obviously a worrying finding, particularly during these times of prolonged social distancing. Well, it can be explained on many levels. You only have to identify the point in the body where people feel loneliness and missing people they are parted from – it's in the centre of the chest where they feel the ache of loneliness and the ache of missing people. 'My heart aches for

you' … 'My heart aches for some company.' I will explain this more in the chapter Escaping Wetiko, but when you realise that the body is the mind – they are expressions of each other – the reason why state of the mind dictates state of the body becomes clear.

American psychologist Ranjit Powar was highlighting the effects of lockdown isolation as early as April, 2020. She said humans have evolved to be social creatures and are wired to live in interactive groups. Being isolated from family, friends and colleagues could be unbalancing and traumatic for most people and could result in short or even long-term psychological and physical health problems. An increase in levels of anxiety, aggression, depression, forgetfulness and hallucinations were possible psychological effects of isolation. 'Mental conditions may be precipitated for those with underlying pre-existing susceptibilities and show up in many others without any pre-condition.' Powar said personal relationships helped us cope with stress and if we lost this outlet for letting off steam the result can be a big emotional void which, for an average person, was difficult to deal with. 'Just a few days of isolation can cause increased levels of anxiety and depression' – so what the hell has been the effect on the global population of *18 months* of this at the time of writing? Powar said: 'Add to it the looming threat of a dreadful disease being repeatedly hammered in through the media and you have a recipe for many shades of mental and physical distress.' For those with a house and a garden it is easy to forget that billions have had to endure lockdown isolation in tiny overcrowded flats and apartments with nowhere to go outside. The psychological and physical consequences of this are unimaginable and with lunatic and abusive partners and parents the consequences have led to tremendous increases in domestic and child abuse and alcoholism as people seek to shut out the horror. Ranjit Powar said:

Staying in a confined space with family is not all a rosy picture for everyone. It can be extremely oppressive and claustrophobic for large low-income families huddled together in small single-room houses. Children here are not lucky enough to have many board/electronic games or books to keep them occupied.

Add to it the deep insecurity of running out of funds for food and basic necessities. On the other hand, there are people with dysfunctional family dynamics, such as domineering, abusive or alcoholic partners, siblings or parents which makes staying home a period of trial. Incidence of suicide and physical abuse against women has shown a worldwide increase. Heightened anxiety and depression also affect a person's immune system, making them more susceptible to illness.

To think that Powar's article was published on April 11th, 2020.

Six-feet fantasy

Social (unsocial) distancing demanded that people stay six feet or two metres apart. UK government advisor Robert Dingwall from the New and Emerging Respiratory Virus Threats Advisory Group said in a radio interview that the two-metre rule was 'conjured up out of nowhere' and was not based on science. No, it was not based on *medical* science, but it didn't come out of nowhere. The distance related to *psychological* science. Six feet/two metres was adopted in many countries and we were told by people like the criminal Anthony Fauci and his ilk that it was founded on science. Many schools could not reopen because they did not have the space for six-feet distancing. Then in March, 2021, after a year of six-feet 'science', a study published in the *Journal of Infectious Diseases* involving more than 500,000 students and almost 100,000 staff over 16 weeks revealed no significant difference in 'Covid' cases between six feet and three feet and Fauci changed his tune. Now three feet was okay. There is no difference between six feet and three *inches* when there is no 'virus' and they got away with six feet for psychological reasons for as long as they could. I hear journalists and others talk about 'unintended consequences' of lockdown. They are not *unintended* at all; they have been coldly-calculated for a specific outcome of human control and that's why super-psychopaths like Gates have called for them so vehemently. Super-psychopath psychologists have demanded them and psychopathic or clueless, spineless, politicians have gone along with them by 'following the science'. But it's not science at all. 'Science' is not what is; it's only what people can be manipulated to believe it is. The whole 'Covid' catastrophe is

founded on mind control. Three word or three statement mantras issued by the UK government are a well-known mind control technique and so we've had 'Stay home/protect the NHS/save lives', 'Stay alert/control the virus/save lives' and 'hands/face/space'. One of the most vocal proponents of extreme 'Covid' rules in the UK has been Professor Susan Michie, a member of the British Communist Party, who is not a medical professional. Michie is the director of the Centre for Behaviour Change at University College London. She is a *behavioural psychologist* and another filthy rich 'Marxist' who praised China's draconian lockdown. She was known by fellow students at Oxford University as 'Stalin's nanny' for her extreme Marxism. Michie is an influential member of the UK government's Scientific Advisory Group for Emergencies (SAGE) and behavioural manipulation groups which have dominated 'Covid' policy. She is a consultant adviser to the World Health Organization on 'Covid-19' and behaviour. Why the hell are lockdowns anything to do with her when they are claimed to be about health? Why does a behavioural psychologist from a group charged with changing the behaviour of the public want lockdown, human isolation and mandatory masks? Does that question really need an answer? Michie *absolutely* has to explain herself before a Nuremberg court when humanity takes back its world again and even more so when you see the consequences of masks that she demands are compulsory. This is a Michie classic:

> The benefits of getting primary school children to wear masks is that regardless of what little degree of transmission is occurring in those age groups it could help normalise the practice. Young children wearing masks may be more likely to get their families to accept masks.

Those words alone should carry a prison sentence when you ponder on the callous disregard for children involved and what a statement it makes about the mind and motivations of Susan Michie. What a lovely lady and what she said there encapsulates the mentality of the psychopaths behind the 'Covid' horror. Let us compare what Michie said with a countrywide study in Germany published at researchsquare.com involving 25,000 school children and 17,854 health complaints submitted by parents. Researchers

found that masks are harming children physically, psychologically, and behaviourally with 24 health issues associated with mask wearing. They include: shortness of breath (29.7%); dizziness (26.4%); increased headaches (53%); difficulty concentrating (50%); drowsiness or fatigue (37%); and malaise (42%). Nearly a third of children experienced more sleep issues than before and a quarter developed new fears. Researchers found health issues and other impairments in 68 percent of masked children covering their faces for an average of 4.5 hours a day. Hundreds of those taking part experienced accelerated respiration, tightness in the chest, weakness, and short-term impairment of consciousness. A reminder of what Michie said again:

The benefits of getting primary school children to wear masks is that regardless of what little degree of transmission is occurring in those age groups it could help normalise the practice. Young children wearing masks may be more likely to get their families to accept masks.

Psychopaths in government and psychology now have children and young people – plus all the adults – wearing masks for hours on end while clueless teachers impose the will of the psychopaths on the young they should be protecting. What the hell are parents doing?

Cult lab rats

We have some schools already imposing on students microchipped buzzers that activate when they get 'too close' to their pals in the way they do with lab rats. How apt. To the Cult and its brain-dead servants our children *are* lab rats being conditioned to be unquestioning, dehumanised slaves for the rest of their lives. Children and young people are being weaned and frightened away from the most natural human instincts including closeness and touch. I have tracked in the books over the years how schools were banning pupils from greeting each other with a hug and the whole Cult-induced Me Too movement has terrified men and boys from a relaxed and natural interaction with female friends and work colleagues to the point where many men try never to be in a room

alone with a woman that's not their partner. Airhead celebrities have as always played their virtue-signalling part in making this happen with their gross exaggeration. For every monster like Harvey Weinstein there are at least tens of thousands of men that don't treat women like that; but everyone must be branded the same and policy changed for them as well as the monster. I am going to be using the word 'dehumanise' many times in this chapter because that is what the Cult is seeking to do and it goes very deep as we shall see. Don't let them kid you that social distancing is planned to end one day. That's not the idea. We are seeing more governments and companies funding and producing wearable gadgets to keep people apart and they would not be doing that if this was meant to be short-term. A tech start-up company backed by GCHQ, the British Intelligence and military surveillance headquarters, has created a social distancing wrist sensor that alerts people when they get too close to others. The CIA has also supported tech companies developing similar devices. The wearable sensor was developed by Tended, one of a number of start-up companies supported by GCHQ (see the CIA and DARPA). The device can be worn on the wrist or as a tag on the waistband and will vibrate whenever someone wearing the device breaches social distancing and gets anywhere near natural human contact. The company had a lucky break in that it was developing a distancing sensor when the 'Covid' hoax arrived which immediately provided a potentially enormous market. How fortunate. The government in big-time Cult-controlled Ontario in Canada is investing $2.5 million in wearable contact tracing technology that 'will alert users if they may have been exposed to the Covid-19 in the workplace and will beep or vibrate if they are within six feet of another person'. Facedrive Inc., the technology company behind this, was founded in 2016 with funding from the Ontario Together Fund and obviously they, too, had a prophet on the board of directors. The human surveillance and control technology is called TraceSCAN and would be worn by the human cyborgs in places such as airports, workplaces, construction sites, care homes and … *schools*.

I emphasise schools with children and young people the prime targets. You know what is planned for society as a whole if you keep your eyes on the schools. They have always been places where the state program the next generation of slaves to be its compliant worker-ants – or Woker-ants these days; but in the mist of the 'Covid' madness they have been transformed into mind laboratories on a scale never seen before. Teachers and head teachers are just as programmed as the kids – often more so. Children are kept apart from human interaction by walk lanes, classroom distancing, staggered meal times, masks, and the rolling-out of buzzer systems. Schools are now physically laid out as a laboratory maze for lab-rats. Lunatics at a school in Anchorage, Alaska, who should be prosecuted for child abuse, took away desks and forced children to kneel (know your place) on a mat for five hours a day while wearing a mask and using their chairs as a desk. How this was supposed to impact on a 'virus' only these clinically insane people can tell you and even then it would be clap-trap. The school banned recess (interaction), art classes (creativity), and physical exercise (getting body and mind moving out of inertia). Everyone behind this outrage should be in jail or better still a mental institution. The behavioural manipulators are all for this dystopian approach to schools. Professor Susan Michie, the mind-doctor and British Communist Party member, said it was wrong to say that schools were safe. They had to be made so by 'distancing', masks and ventilation (sitting all day in the cold). I must ask this lady round for dinner on a night I know I am going to be out and not back for weeks. She probably wouldn't be able to make it, anyway, with all the visits to her own psychologist she must have block-booked.

Masking identity

I know how shocking it must be for you that a behaviour manipulator like Michie wants everyone to wear masks which have long been a feature of mind-control programs like the infamous MKUltra in the United States, but, there we are. We live and learn. I spent many years from 1996 to right across the millennium

researching mind control in detail on both sides of the Atlantic and elsewhere. I met a large number of mind-control survivors and many had been held captive in body and mind by MKUltra. MK stands for mind-control, but employs the German spelling in deference to the Nazis spirited out of Germany at the end of World War Two by Operation Paperclip in which the US authorities, with help from the Vatican, transported Nazi mind-controllers and engineers to America to continue their work. Many of them were behind the creation of NASA and they included Nazi scientist and SS officer Wernher von Braun who swapped designing V-2 rockets to bombard London with designing the Saturn V rockets that powered the NASA moon programme's Apollo craft. I think I may have mentioned that the Cult has no borders. Among Paperclip escapees was Josef Mengele, the Angel of Death in the Nazi concentration camps where he conducted mind and genetic experiments on children often using twins to provide a control twin to measure the impact of his 'work' on the other. If you want to observe the Cult mentality in all its extremes of evil then look into the life of Mengele. I have met many people who suffered mercilessly under Mengele in the United States where he operated under the name Dr Greene and became a stalwart of MKUltra programming and torture. Among his locations was the underground facility in the Mojave Desert in California called the China Lake Naval Weapons Station which is almost entirely below the surface. My books *The Biggest Secret*, *Children of the Matrix* and *The Perception Deception* have the detailed background to MKUltra.

The best-known MKUltra survivor is American Cathy O'Brien. I first met her and her late partner Mark Phillips at a conference in Colorado in 1996. Mark helped her escape and deprogram from decades of captivity in an offshoot of MKUltra known as Project Monarch in which 'sex slaves' were provided for the rich and famous including Father George Bush, Dick Cheney and the Clintons. Read Cathy and Mark's book *Trance-Formation of America* and if you are new to this you will be shocked to the core. I read it in 1996 shortly before, with the usual synchronicity of my life, I found

myself given a book table at the conference right next to hers. MKUltra never ended despite being very publicly exposed (only a small part of it) in the 1970s and continues in other guises. I am still in touch with Cathy. She contacted me during 2020 after masks became compulsory in many countries to tell me how they were used as part of MKUltra programming. I had been observing 'Covid regulations' and the relationship between authority and public for months. I saw techniques that I knew were employed on individuals in MKUltra being used on the global population. I had read many books and manuals on mind control including one called *Silent Weapons for Quiet Wars* which came to light in the 1980s and was a guide on how to perceptually program on a mass scale. 'Silent Weapons' refers to mind-control. I remembered a line from the manual as governments, medical authorities and law enforcement agencies have so obviously talked to – or rather at – the adult population since the 'Covid' hoax began as if they are children. The document said:

> If a person is spoken to by a T.V. advertiser as if he were a twelve-year-old, then, due to suggestibility, he will, with a certain probability, respond or react to that suggestion with the uncritical response of a twelve-year-old and will reach in to his economic reservoir and deliver its energy to buy that product on impulse when he passes it in the store.

That's why authority has spoken to adults like children since all this began.

Why did Michael Jackson wear masks?

Every aspect of the 'Covid' narrative has mind-control as its central theme. Cathy O'Brien wrote an article for davidicke.com about the connection between masks and mind control. Her daughter Kelly who I first met in the 1990s was born while Cathy was still held captive in MKUltra. Kelly was forced to wear a mask as part of her programming from the age of *two* to dehumanise her, target her sense of individuality and reduce the amount of oxygen her brain and body received. *Bingo*. This is the real reason for compulsory

masks, why they have been enforced en masse, and why they seek to increase the number they demand you wear. First one, then two, with one disgraceful alleged 'doctor' recommending four which is nothing less than a death sentence. Where and how often they must be worn is being expanded for the purpose of mass mind control and damaging respiratory health which they can call 'Covid-19'. Canada's government headed by the man-child Justin Trudeau, says it's fine for children of two and older to wear masks. An insane 'study' in Italy involving just 47 children concluded there was no problem for babies as young as *four months* wearing them. Even after people were 'vaccinated' they were still told to wear masks by the criminal that is Anthony Fauci. Cathy wrote that mandating masks is allowing the authorities literally to control the air we breathe which is what was done in MKUltra. You might recall how the singer Michael Jackson wore masks and there is a reason for that. He was subjected to MKUltra mind control through Project Monarch and his psyche was scrambled by these simpletons. Cathy wrote:

In MKUltra Project Monarch mind control, Michael Jackson had to wear a mask to silence his voice so he could not reach out for help. Remember how he developed that whisper voice when he wasn't singing? Masks control the mind from the outside in, like the redefining of words is doing. By controlling what we can and cannot say for fear of being labeled racist or beaten, for example, it ultimately controls thought that drives our words and ultimately actions (or lack thereof).

Likewise, a mask muffles our speech so that we are not heard, which controls voice … words … mind. This is Mind Control. Masks are an obvious mind control device, and I am disturbed so many people are complying on a global scale. Masks depersonalize while making a person feel as though they have no voice. It is a barrier to others. People who would never choose to comply but are forced to wear a mask in order to keep their job, and ultimately their family fed, are compromised. They often feel shame and are subdued. People have stopped talking with each other while media controls the narrative.

The 'no voice' theme has often become literal with train passengers told not to speak to each other in case they pass on the 'virus', singing banned for the same reason and bonkers California officials telling people riding roller coasters that they cannot shout and scream. Cathy said she heard every day from healed MKUltra survivors who cannot wear a mask without flashing back on ways

their breathing was controlled – 'from ball gags and penises to water boarding'. She said that through the years when she saw images of people in China wearing masks 'due to pollution' that it was really to control their oxygen levels. 'I knew it was as much of a population control mechanism of depersonalisation as are burkas', she said. Masks are another Chinese communist/fascist method of control that has been swept across the West as the West becomes China at lightning speed since we entered 2020.

Mask-19

There are other reasons for mandatory masks and these include destroying respiratory health to call it 'Covid-19' and stunting brain development of children and the young. Dr Margarite Griesz-Brisson MD, PhD, is a Consultant Neurologist and Neurophysiologist and the Founder and Medical Director of the London Neurology and Pain Clinic. Her CV goes down the street and round the corner. She is clearly someone who cares about people and won't parrot the propaganda. Griesz-Brisson has a PhD in pharmacology, with special interest in neurotoxicology, environmental medicine, neuroregeneration and neuroplasticity (the way the brain can change in the light of information received). She went public in October, 2020, with a passionate warning about the effects of mask-wearing laws:

> The reinhalation of our exhaled air will without a doubt create oxygen deficiency and a flooding of carbon dioxide. We know that the human brain is very sensitive to oxygen deprivation. There are nerve cells for example in the hippocampus that can't be longer than 3 minutes without oxygen – they cannot survive. The acute warning symptoms are headaches, drowsiness, dizziness, issues in concentration, slowing down of reaction time – reactions of the cognitive system.

Oh, I know, let's tell bus, truck and taxi drivers to wear them and people working machinery. How about pilots, doctors and police? Griesz-Brisson makes the important point that while the symptoms she mentions may fade as the body readjusts this does not alter the fact that people continue to operate in oxygen deficit with long list of

potential consequences. She said it was well known that neurodegenerative diseases take years or decades to develop. 'If today you forget your phone number, the breakdown in your brain would have already started 20 or 30 years ago.' She said degenerative processes in your brain are getting amplified as your oxygen deprivation continues through wearing a mask. Nerve cells in the brain are unable to divide themselves normally in these circumstances and lost nerve cells will no longer be regenerated. 'What is gone is gone.' Now consider that people like shop workers and *schoolchildren* are wearing masks for hours every day. What in the name of sanity is going to be happening to them? 'I do not wear a mask, I need my brain to think', Griesz-Brisson said, 'I want to have a clear head when I deal with my patients and not be in a carbon dioxide-induced anaesthesia'. If you are told to wear a mask anywhere ask the organisation, police, store, whatever, for their risk assessment on the dangers and negative effects on mind and body of enforcing mask-wearing. They won't have one because it has never been done not even by government. All of them must be subject to class-action lawsuits as the consequences come to light. They don't do mask risk assessments for an obvious reason. They know what the conclusions would be and independent scientific studies that *have* been done tell a horror story of consequences.

'Masks are criminal'

Dr Griesz-Brisson said that for children and adolescents, masks are an absolute no-no. They had an extremely active and adaptive immune system and their brain was incredibly active with so much to learn. 'The child's brain, or the youth's brain, is thirsting for oxygen.' The more metabolically active an organ was, the more oxygen it required; and in children and adolescents every organ was metabolically active. Griesz-Brisson said that to deprive a child's or adolescent's brain of oxygen, or to restrict it in any way, was not only dangerous to their health, it was absolutely criminal. 'Oxygen deficiency inhibits the development of the brain, and the damage that has taken place as a result CANNOT be reversed.' Mind

manipulators of MKUltra put masks on two-year-olds they wanted to neurologically rewire and you can see why. Griesz-Brisson said a child needs the brain to learn and the brain needs oxygen to function. 'We don't need a clinical study for that. This is simple, indisputable physiology.' Consciously and purposely induced oxygen deficiency was an absolutely deliberate health hazard, and an absolute medical contraindication which means that 'this drug, this therapy, this method or measure should not be used, and is not allowed to be used'. To coerce an entire population to use an absolute medical contraindication by force, she said, there had to be definite and serious reasons and the reasons must be presented to competent interdisciplinary and independent bodies to be verified and authorised. She had this warning of the consequences that were coming if mask wearing continued:

When, in ten years, dementia is going to increase exponentially, and the younger generations couldn't reach their god-given potential, it won't help to say 'we didn't need the masks'. I know how damaging oxygen deprivation is for the brain, cardiologists know how damaging it is for the heart, pulmonologists know how damaging it is for the lungs. Oxygen deprivation damages every single organ. Where are our health departments, our health insurance, our medical associations? It would have been their duty to be vehemently against the lockdown and to stop it and stop it from the very beginning.

Why do the medical boards issue punishments to doctors who give people exemptions? Does the person or the doctor seriously have to prove that oxygen deprivation harms people? What kind of medicine are our doctors and medical associations representing? Who is responsible for this crime? The ones who want to enforce it? The ones who let it happen and play along, or the ones who don't prevent it?

All of the organisations and people she mentions there either answer directly to the Cult or do whatever hierarchical levels above them tell them to do. The outcome of both is the same. 'It's not about masks, it's not about viruses, it's certainly not about your health', Griesz-Brisson said. 'It is about much, much more. I am not participating. I am not afraid.' They were taking our air to breathe and there was no unfounded medical exemption from face masks. Oxygen deprivation was dangerous for every single brain. It had to be the free decision of every human being whether they want to

wear a mask that was absolutely ineffective to protect themselves from a virus. She ended by rightly identifying where the responsibility lies for all this:

> The imperative of the hour is personal responsibility. We are responsible for what we think, not the media. We are responsible for what we do, not our superiors. We are responsible for our health, not the World Health Organization. And we are responsible for what happens in our country, not the government.

Halle-bloody-lujah.

But surgeons wear masks, right?

Independent studies of mask-wearing have produced a long list of reports detailing mental, emotional and physical dangers. What a definition of insanity to see police officers imposing mask-wearing on the public which will cumulatively damage their health while the police themselves wear masks that will cumulatively damage *their* health. It's utter madness and both public and police do this because 'the government says so' – yes a government of brain-donor idiots like UK Health Secretary Matt Hancock reading the 'follow the science' scripts of psychopathic, lunatic psychologists. The response you get from Stockholm syndrome sufferers defending the very authorities that are destroying them and their families is that 'surgeons wear masks'. This is considered the game, set and match that they must work and don't cause oxygen deficit. Well, actually, scientific studies have shown that they *do* and oxygen levels are monitored in operating theatres to compensate. Surgeons wear masks to stop spittle and such like dropping into open wounds – not to stop 'viral particles' which are so miniscule they can only be seen through an electron microscope. Holes in the masks are significantly bigger than 'viral particles' and if you sneeze or cough they will breach the mask. I watched an incredibly disingenuous 'experiment' that claimed to prove that masks work in catching 'virus' material from the mouth and nose. They did this with a slow motion camera and the mask did block big stuff which stayed inside the mask and

against the face to be breathed in or cause infections on the face as we have seen with many children. 'Viral particles', however, would never have been picked up by the camera as they came through the mask when they are far too small to be seen. The 'experiment' was therefore disingenuous *and* useless.

Studies have concluded that wearing masks in operating theatres (and thus elsewhere) make no difference to preventing infection while the opposite is true with toxic shite building up in the mask and this had led to an explosion in tooth decay and gum disease dubbed by dentists 'mask mouth'. You might have seen the Internet video of a furious American doctor urging people to take off their masks after a four-year-old patient had been rushed to hospital the night before and nearly died with a lung infection that doctors sourced to mask wearing. A study in the journal *Cancer Discovery* found that inhalation of harmful microbes can contribute to advanced stage lung cancer in adults and long-term use of masks can help breed dangerous pathogens. Microbiologists have said frequent mask wearing creates a moist environment in which microbes can grow and proliferate before entering the lungs. The Canadian Agency for Drugs and Technologies in Health, or CADTH, a Canadian national organisation that provides research and analysis to healthcare decision-makers, said this as long ago as 2013 in a report entitled 'Use of Surgical Masks in the Operating Room: A Review of the Clinical Effectiveness and Guidelines'. It said:

- No evidence was found to support the use of surgical face masks to reduce the frequency of surgical site infections
- No evidence was found on the effectiveness of wearing surgical face masks to protect staff from infectious material in the operating room.
- Guidelines recommend the use of surgical face masks by staff in the operating room to protect both operating room staff and patients (despite the lack of evidence).

We were told that the world could go back to 'normal' with the arrival of the 'vaccines'. When they came, fraudulent as they are, the story changed as I knew that it would. We are in the midst of transforming 'normal', not going back to it. Mary Ramsay, head of immunisation at Public Health England, echoed the words of US criminal Anthony Fauci who said masks and other regulations must stay no matter if people are vaccinated. The Fauci idiot continued to wear two masks – different colours so both could be clearly seen – after he *claimed* to have been vaccinated. Senator Rand Paul told Fauci in one exchange that his double-masks were 'theatre' and he was right. It's all theatre. Mary Ramsay back-tracked on the vaccine-return-to-normal theme when she said the public may need to wear masks and social-distance for years despite the jabs. 'People have got used to those lower-level restrictions now, and [they] can live with them', she said telling us what the idea has been all along. 'The vaccine does not give you a pass, even if you have had it, you must continue to follow all the guidelines' said a Public Health England statement which reneged on what we had been told before and made having the 'vaccine' irrelevant to 'normality' even by the official story. Spain's fascist government trumped everyone by passing a law mandating the wearing of masks on the beach and even when swimming in the sea. The move would have devastated what's left of the Spanish tourist industry, posed potential breathing dangers to swimmers and had Northern European sunbathers walking around with their forehead brown and the rest of their face white as a sheet. The ruling was so crazy that it had to be retracted after pressure from public and tourist industry, but it confirmed where the Cult wants to go with masks and how clinically insane authority has become. The determination to make masks permanent and hide the serious dangers to body and mind can be seen in the censorship of scientist Professor Denis Rancourt by Bill Gates-funded academic publishing website ResearchGate over his papers exposing the dangers and uselessness of masks. Rancourt said:

ResearchGate today has permanently locked my account, which I have had since 2015. Their reasons graphically show the nature of their attack against democracy, and their corruption of

science … By their obscene non-logic, a scientific review of science articles reporting on harms caused by face masks has a 'potential to cause harm'. No criticism of the psychological device (face masks) is tolerated, if the said criticism shows potential to influence public policy.

This is what happens in a fascist world.

Where are the 'greens' (again)?

Other dangers of wearing masks especially regularly relate to the inhalation of minute plastic fibres into the lungs and the deluge of discarded masks in the environment and oceans. Estimates predicted that more than 1.5 billion disposable masks will end up in the world's oceans every year polluting the water with tons of plastic and endangering marine wildlife. Studies project that humans are using 129 billion face masks each month worldwide – about three million a minute. Most are disposable and made from plastic, non-biodegradable microfibers that break down into smaller plastic particles that become widespread in ecosystems. They are littering cities, clogging sewage channels and turning up in bodies of water. I have written in other books about the immense amounts of microplastics from endless sources now being absorbed into the body. Rolf Halden, director of the Arizona State University (ASU) Biodesign Center for Environmental Health Engineering, was the senior researcher in a 2020 study that analysed 47 human tissue samples and found microplastics in all of them. 'We have detected these chemicals of plastics in every single organ that we have investigated', he said. I wrote in *The Answer* about the world being deluged with microplastics. A study by the Worldwide Fund for Nature (WWF) found that people are consuming on average every week some 2,000 tiny pieces of plastic mostly through water and also through marine life and the air. Every year humans are ingesting enough microplastics to fill a heaped dinner plate and in a life-time of 79 years it is enough to fill two large waste bins. Marco Lambertini, WWF International director general said: 'Not only are plastics polluting our oceans and waterways and killing marine life – it's in all of us and we can't escape consuming plastics,' American

geologists found tiny plastic fibres, beads and shards in rainwater samples collected from the remote slopes of the Rocky Mountain National Park near Denver, Colorado. Their report was headed: 'It is raining plastic.' Rachel Adams, senior lecturer in Biomedical Science at Cardiff Metropolitan University, said that among health consequences are internal inflammation and immune responses to a 'foreign body'. She further pointed out that microplastics become carriers of toxins including mercury, pesticides and dioxins (a known cause of cancer and reproductive and developmental problems). These toxins accumulate in the fatty tissues once they enter the body through microplastics. Now this is being compounded massively by people putting plastic on their face and throwing it away.

Workers exposed to polypropylene plastic fibres known as 'flock' have developed 'flock worker's lung' from inhaling small pieces of the flock fibres which can damage lung tissue, reduce breathing capacity and exacerbate other respiratory problems. *Now* … commonly used surgical masks have three layers of melt-blown textiles made of … polypropylene. We have billions of people putting these microplastics against their mouth, nose and face for hours at a time day after day in the form of masks. How does anyone think that will work out? I mean – what could possibly go wrong? We posted a number of scientific studies on this at davidicke.com, but when I went back to them as I was writing this book the links to the science research website where they were hosted were dead. Anything that challenges the official narrative in any way is either censored or vilified. The official narrative is so unsupportable by the evidence that only deleting the truth can protect it. A study by Chinese scientists still survived – with the usual twist which it why it was still active, I guess. Yes, they found that virtually all the masks they tested increased the daily intake of microplastic fibres, but people should still wear them because the danger from the 'virus' was worse said the crazy 'team' from the Institute of Hydrobiology in Wuhan. Scientists first discovered microplastics in lung tissue of some patients who died of lung cancer

in the 1990s. Subsequent studies have confirmed the potential health damage with the plastic degrading slowly and remaining in the lungs to accumulate in volume. Wuhan researchers used a machine simulating human breathing to establish that masks shed up to nearly 4,000 microplastic fibres in a month with reused masks producing more. Scientists said some masks are laced with toxic chemicals and a variety of compounds seriously restricted for both health and environmental reasons. They include cobalt (used in blue dye) and formaldehyde known to cause watery eyes, burning sensations in the eyes, nose, and throat, plus coughing, wheezing and nausea. No – that must be 'Covid-19'.

Mask 'worms'

There is another and potentially even more sinister content of masks. Mostly new masks of different makes filmed under a microscope around the world have been found to contain strange black fibres or 'worms' that appear to move or 'crawl' by themselves and react to heat and water. The nearest I have seen to them are the self-replicating fibres that are pulled out through the skin of those suffering from Morgellons disease which has been connected to the phenomena of 'chemtrails' which I will bring into the story later on. Morgellons fibres continue to grow outside the body and have a form of artificial intelligence. Black 'worm' fibres in masks have that kind of feel to them and there is a nanotechnology technique called 'worm micelles' which carry and release drugs or anything else you want to deliver to the body. For sure the suppression of humanity by mind altering drugs is the Cult agenda big time and the more excuses they can find to gain access to the body the more opportunities there are to make that happen whether through 'vaccines' or masks pushed against the mouth and nose for hours on end.

So let us summarise the pros and cons of masks:

Against masks: Breathing in your own carbon dioxide; depriving the body and brain of sufficient oxygen; build-up of toxins in the mask that can be breathed into the lungs and cause rashes on the face and 'mask-mouth'; breathing microplastic fibres and toxic chemicals into the lungs; dehumanisation and deleting individualisation by literally making people faceless; destroying human emotional interaction through facial expression and deleting parental connection with their babies which look for guidance to their facial expression.

For masks: They don't protect you from a 'virus' that doesn't exist and even if it did 'viral' particles are so minute they are smaller than the holes in the mask.

Governments, police, supermarkets, businesses, transport companies, and all the rest who seek to impose masks have done no risk assessment on their consequences for health and psychology and are now open to group lawsuits when the impact becomes clear with a cumulative epidemic of respiratory and other disease. Authorities will try to exploit these effects and hide the real cause by dubbing them 'Covid-19'. Can you imagine setting out to force the population to wear health-destroying masks without doing any assessment of the risks? It is criminal and it is evil, but then how many people targeted in this way, who see their children told to wear them all day at school, have asked for a risk assessment? Billions can't be imposed upon by the few unless the billions allow it. Oh, yes, with just a tinge of irony, 85 percent of all masks made worldwide come from *China*.

Wash your hands in toxic shite

'Covid' rules include the use of toxic sanitisers and again the health consequences of constantly applying toxins to be absorbed through the skin is obvious to any level of Renegade Mind. America's Food and Drug Administration (FDA) said that sanitisers are drugs and issued a warning about 75 dangerous brands which contain

methanol used in antifreeze and can cause death, kidney damage and blindness. The FDA circulated the following warning even for those brands that it claims to be safe:

Store hand sanitizer out of the reach of pets and children, and children should use it only with adult supervision. Do not drink hand sanitizer. This is particularly important for young children, especially toddlers, who may be attracted by the pleasant smell or brightly colored bottles of hand sanitizer.

Drinking even a small amount of hand sanitizer can cause alcohol poisoning in children. (However, there is no need to be concerned if your children eat with or lick their hands after using hand sanitizer.) During this coronavirus pandemic, poison control centers have had an increase in calls about accidental ingestion of hand sanitizer, so it is important that adults monitor young children's use.

Do not allow pets to swallow hand sanitizer. If you think your pet has eaten something potentially dangerous, call your veterinarian or a pet poison control center right away. Hand sanitizer is flammable and should be stored away from heat and flames. When using hand sanitizer, rub your hands until they feel completely dry before performing activities that may involve heat, sparks, static electricity, or open flames.

There you go, perfectly safe, then, and that's without even a mention of the toxins absorbed through the skin. Come on kids – sanitise your hands everywhere you go. It will save you from the 'virus'. Put all these elements together of the 'Covid' normal and see how much health and psychology is being cumulatively damaged, even devastated, to 'protect your health'. Makes sense, right? They are only imposing these things because they care, right? *Right?*

Submitting to insanity

Psychological reframing of the population goes very deep and is done in many less obvious ways. I hear people say how contradictory and crazy 'Covid' rules are and how they are ever changing. This is explained away by dismissing those involved as idiots. It is a big mistake. The Cult is delighted if its cold calculation is perceived as incompetence and idiocy when it is anything but. Oh, yes, there are idiots within the system – lots of them – but they are *administering* the Cult agenda, mostly unknowingly. They are not deciding and dictating it. The bulwark against tyranny is self-

respect, always has been, always will be. It is self-respect that has broken every tyranny in history. By its very nature self-respect will not bow to oppression and its perpetrators. There is so little self-respect that it's always the few that overturn dictators. Many may eventually follow, but the few with the iron spines (self-respect) kick it off and generate the momentum. The Cult targets self-respect in the knowledge that once this has gone only submission remains. Crazy, contradictory, ever-changing 'Covid' rules are systematically applied by psychologists to delete self-respect. They *want* you to see that the rules make no sense. It is one thing to decide to do something when *you* have made the choice based on evidence and logic. You still retain your self-respect. It is quite another when you can see what you are being told to do is insane, ridiculous and makes no sense, and *yet you still do it*. Your self-respect is extinguished and this has been happening as ever more obviously stupid and nonsensical things have been demanded and the great majority have complied even when they can see they are stupid and nonsensical.

People walk around in face-nappies knowing they are damaging their health and make no difference to a 'virus'. They do it in fear of not doing it. I know it's daft, but I'll do it anyway. When that happens something dies inside of you and submissive reframing has begun. Next there's a need to hide from yourself that you have conceded your self-respect and you convince yourself that you have not really submitted to fear and intimidation. You begin to believe that you are complying with craziness because it's the right thing to do. When first you concede your self-respect of 2+2 = 4 to 2+2 = 5 you *know* you are compromising your self-respect. Gradually to avoid facing that fact you begin to *believe* that 2+2=5. You have been reframed and I have been watching this process happening in the human psyche on an industrial scale. The Cult is working to break your spirit and one of its major tools in that war is humiliation. I read how former American soldier Bradley Manning (later Chelsea Manning after a sex-change) was treated after being jailed for supplying WikiLeaks with documents exposing the enormity of

government and elite mendacity. Manning was isolated in solitary confinement for eight months, put under 24-hour surveillance, forced to hand over clothing before going to bed, and stand naked for every roll call. This is systematic humiliation. The introduction of anal swab 'Covid' tests in China has been done for the same reason to delete self-respect and induce compliant submission. Anal swabs are mandatory for incoming passengers in parts of China and American diplomats have said they were forced to undergo the indignity which would have been calculated humiliation by the Cult-owned Chinese government that has America in its sights.

Government-people: An abusive relationship

Spirit-breaking psychological techniques include giving people hope and apparent respite from tyranny only to take it away again. This happened in the UK during Christmas, 2020, when the psycho-psychologists and their political lackeys announced an easing of restrictions over the holiday only to reimpose them almost immediately on the basis of yet another lie. There is a big psychological difference between getting used to oppression and being given hope of relief only to have that dashed. Psychologists know this and we have seen the technique used repeatedly. Then there is traumatising people before you introduce more extreme regulations that require compliance. A perfect case was the announcement by the dark and sinister Whitty and Vallance in the UK that 'new data' predicted that 4,000 could die every day over the winter of 2020/2021 if we did not lockdown again. I think they call it lying and after traumatising people with that claim out came Jackboot Johnson the next day with new curbs on human freedom. Psychologists know that a frightened and traumatised mind becomes suggestable to submission and behaviour reframing. Underpinning all this has been to make people fearful and suspicious of each other and see themselves as a potential danger to others. In league with deleted self-respect you have the perfect psychological recipe for self-loathing. The relationship between authority and public is now demonstrably the same as that of

subservience to an abusive partner. These are signs of an abusive relationship explained by psychologist Leslie Becker-Phelps:

Psychological and emotional abuse: Undermining a partner's self-worth with verbal attacks, name-calling, and belittling. Humiliating the partner in public, unjustly accusing them of having an affair, or interrogating them about their every behavior. Keeping partner confused or off balance by saying they were just kidding or blaming the partner for 'making' them act this way … Feigning in public that they care while turning against them in private. This leads to victims frequently feeling confused, incompetent, unworthy, hopeless, and chronically self-doubting. [Apply these techniques to how governments have treated the population since New Year, 2020, and the parallels are obvious.]

Physical abuse: The abuser might physically harm their partner in a range of ways, such as grabbing, hitting, punching, or shoving them. They might throw objects at them or harm them with a weapon. [Observe the physical harm imposed by masks, lockdown, and so on.]

Threats and intimidation: One way abusers keep their partners in line is by instilling fear. They might be verbally threatening, or give threatening looks or gestures. Abusers often make it known that they are tracking their partner's every move. They might destroy their partner's possessions, threaten to harm them, or threaten to harm their family members. Not surprisingly, victims of this abuse often feel anxiety, fear, and panic. [No words necessary.]

Isolation: Abusers often limit their partner's activities, forbidding them to talk or interact with friends or family. They might limit access to a car or even turn off their phone. All of this might be done by physically holding them against their will, but is often accomplished through psychological abuse and intimidation. The more isolated a person feels, the fewer resources they have to help gain perspective on their situation and to escape from it. [No words necessary.]

Economic abuse: Abusers often make their partners beholden to them for money by controlling access to funds of any kind. They might prevent their partner from getting a job or withhold access to money they earn from a job. This creates financial dependency that makes leaving the relationship very difficult. [See destruction of livelihoods and the proposed meagre 'guaranteed income' so long as you do whatever you are told.]

Using children: An abuser might disparage their partner's parenting skills, tell their children lies about their partner, threaten to take custody of their children, or threaten to harm their children. These tactics instil fear and often elicit compliance. [See reframed social service mafia and how children are being mercilessly abused by the state over 'Covid' while their parents look on too frightened to do anything.]

A further recurring trait in an abusive relationship is the abused blaming themselves for their abuse and making excuses for the abuser. We have the public blaming each other for lockdown abuse by government and many making excuses for the government while attacking those who challenge the government. How often we have heard authorities say that rules are being imposed or reimposed only because people have refused to 'behave' and follow the rules. We don't want to do it – it's *you*.

Renegade Minds are an antidote to all of these things. They will never concede their self-respect no matter what the circumstances. Even when apparent humiliation is heaped upon them they laugh in its face and reflect back the humiliation on the abuser where it belongs. Renegade Minds will never wear masks they know are only imposed to humiliate, suppress and damage both physically and psychologically. Consequences will take care of themselves and they will never break their spirit or cause them to concede to tyranny. UK newspaper columnist Peter Hitchens was one of the few in the mainstream media to speak out against lockdowns and forced vaccinations. He then announced he had taken the jab. He wanted to see family members abroad and he believed vaccine passports were inevitable even though they had not yet been introduced. Hitchens

has a questioning and critical mind, but not a Renegade one. If he had no amount of pressure would have made him concede. Hitchens excused his action by saying that the battle has been lost. Renegade Minds never accept defeat when freedom is at stake and even if they are the last one standing the self-respect of not submitting to tyranny is more important than any outcome or any consequence.

That's why Renegade Minds are the only minds that ever changed anything worth changing.

CHAPTER EIGHT

'Reframing' insanity

Insanity is relative. It depends on who has who locked in what cage
Ray Bradbury

'Reframing' a mind means simply to change its perception and behaviour. This can be done subconsciously to such an extent that subjects have no idea they have been 'reframed' while to any observer changes in behaviour and attitudes are obvious.

Human society is being reframed on a ginormous scale since the start of 2020 and here we have the reason why psychologists rather than doctors have been calling the shots. Ask most people who have succumbed to 'Covid' reframing if they have changed and most will say 'no'; but they *have* and fundamentally. The Cult's long-game has been preparing for these times since way back and crucial to that has been to prepare both population and officialdom mentally and emotionally. To use the mind-control parlance they had to reframe the population with a mentality that would submit to fascism and reframe those in government and law enforcement to impose fascism or at least go along with it. The result has been the fact-deleted mindlessness of 'Wokeness' and officialdom that has either enthusiastically or unquestioningly imposed global tyranny demanded by reframed politicians on behalf of psychopathic and deeply evil cultists. 'Cognitive reframing' identifies and challenges the way someone sees the world in the form of situations, experiences and emotions and then restructures those perceptions to view the same set of circumstances in a different way. This can have

benefits if the attitudes are personally destructive while on the other side it has the potential for individual and collective mind control which the subject has no idea has even happened.

Cognitive therapy was developed in the 1960s by Aaron T. Beck who was born in Rhode Island in 1921 as the son of Jewish immigrants from the Ukraine. He became interested in the techniques as a treatment for depression. Beck's daughter Judith S. Beck is prominent in the same field and they founded the Beck Institute for Cognitive Behavior Therapy in Philadelphia in 1994. Cognitive reframing, however, began to be used worldwide by those with a very dark agenda. The Cult reframes politicians to change their attitudes and actions until they are completely at odds with what they once appeared to stand for. The same has been happening to government administrators at all levels, law enforcement, military and the human population. Cultists love mind control for two main reasons: It allows them to control what people think, do and say to secure agenda advancement and, by definition, it calms their legendary insecurity and fear of the unexpected. I have studied mind control since the time I travelled America in 1996. I may have been talking to next to no one in terms of an audience in those years, but my goodness did I gather a phenomenal amount of information and knowledge about so many things including the techniques of mind control. I have described this in detail in other books going back to *The Biggest Secret* in 1998. I met a very large number of people recovering from MKUltra and its offshoots and successors and I began to see how these same techniques were being used on the population in general. This was never more obvious than since the 'Covid' hoax began.

Reframing the enforcers

I have observed over the last two decades and more the very clear transformation in the dynamic between the police, officialdom and the public. I tracked this in the books as the relationship mutated from one of serving the public to seeing them as almost the enemy and certainly a lower caste. There has always been a class divide

based on income and always been some psychopathic, corrupt, and big-I-am police officers. This was different. Wholesale change was unfolding in the collective dynamic; it was less about money and far more about position and perceived power. An us-and-them was emerging. Noses were lifted skyward by government administration and law enforcement and their attitude to the public they were *supposed* to be serving changed to one of increasing contempt, superiority and control. The transformation was so clear and widespread that it had to be planned. Collective attitudes and dynamics do not change naturally and organically that quickly on that scale. I then came across an organisation in Britain called Common Purpose created in the late 1980s by Julia Middleton who would work in the office of Deputy Prime Minister John Prescott during the long and disastrous premiership of war criminal Tony Blair. When Blair speaks the Cult is speaking and the man should have been in jail a long time ago. Common Purpose proclaims itself to be one of the biggest 'leadership development' organisations in the world while functioning as a *charity* with all the financial benefits which come from that. It hosts 'leadership development' courses and programmes all over the world and claims to have 'brought together' what it calls 'leaders' from more than 100 countries on six continents. The modus operandi of Common Purpose can be compared with the work of the UK government's reframing network that includes the Behavioural Insights Team 'nudge unit' and 'Covid' reframing specialists at SPI-B. WikiLeaks described Common Purpose long ago as 'a hidden virus in our government and schools' which is unknown to the general public: 'It recruits and trains "leaders" to be loyal to the directives of Common Purpose and the EU, instead of to their own departments, which they then undermine or subvert, the NHS [National Health Service] being an example.' This is a vital point to understand the 'Covid' hoax. The NHS, and its equivalent around the world, has been utterly reframed in terms of administrators and much of the medical personnel with the transformation underpinned by recruitment policies. The outcome has been the criminal and psychopathic behaviour of the

NHS over 'Covid' and we have seen the same in every other major country. WikiLeaks said Common Purpose trainees are 'learning to rule without regard to democracy' and to usher in a police state (current events explained). Common Purpose operated like a 'glue' and had members in the NHS, BBC, police, legal profession, church, many of Britain's 7,000 quangos, local councils, the Civil Service, government ministries and Parliament, and controlled many RDA's (Regional Development Agencies). Here we have one answer for how and why British institutions and their like in other countries have changed so negatively in relation to the public. This further explains how and why the beyond-disgraceful reframed BBC has become a propaganda arm of 'Covid' fascism. They are all part of a network pursuing the same goal.

By 2019 Common Purpose was quoting a figure of 85,000 'leaders' that had attended its programmes. These 'students' of all ages are known as Common Purpose 'graduates' and they consist of government, state and local government officials and administrators, police chiefs and officers, and a whole range of others operating within the national, local and global establishment. Cressida Dick, Commissioner of the London Metropolitan Police, is the Common Purpose graduate who was the 'Gold Commander' that oversaw what can only be described as the murder of Brazilian electrician Jean Charles de Menezes in 2005. He was held down by psychopathic police and shot seven times in the head by a psychopathic lunatic after being mistaken for a terrorist when he was just a bloke going about his day. Dick authorised officers to pursue and keep surveillance on de Menezes and ordered that he be stopped from entering the underground train system. Police psychopaths took her at her word clearly. She was 'disciplined' for this outrage by being *promoted* – eventually to the top of the 'Met' police where she has been a disaster. Many Chief Constables controlling the police in different parts of the UK are and have been Common Purpose graduates. I have heard the 'graduate' network described as a sort of Mafia or secret society operating within the fabric of government at all levels pursuing a collective policy

ingrained at Common Purpose training events. Founder Julia Middleton herself has said:

> Locally and internationally, Common Purpose graduates will be 'lighting small fires' to create change in their organisations and communities … The Common Purpose effect is best illustrated by the many stories of small changes brought about by leaders, who themselves have changed.

A Common Purpose mission statement declared:

> Common Purpose aims to improve the way society works by expanding the vision, decision-making ability and influence of all kinds of leaders. The organisation runs a variety of educational programmes for leaders of all ages, backgrounds and sectors, in order to provide them with the inspirational, information and opportunities they need to change the world.

Yes, but into what? Since 2020 the answer has become clear.

NLP and the Delphi technique

Common Purpose would seem to be a perfect name or would common programming be better? One of the foundation methods of reaching 'consensus' (group think) is by setting the agenda theme and then encouraging, cajoling or pressuring everyone to agree a 'consensus' in line with the core theme promoted by Common Purpose. The methodology involves the 'Delphi technique', or an adaption of it, in which opinions are expressed that are summarised by a 'facilitator or change agent' at each stage. Participants are 'encouraged' to modify their views in the light of what others have said. Stage by stage the former individual opinions are merged into group consensus which just happens to be what Common Purpose wants them to believe. A key part of this is to marginalise anyone refusing to concede to group think and turn the group against them to apply pressure to conform. We are seeing this very technique used on the general population to make 'Covid' group-thinkers hostile to those who have seen through the bullshit. People can be reframed by using perception manipulation methods such as Neuro-Linguistic Programming (NLP) in which you change perception with the use of

carefully constructed language. An NLP website described the technique this way:

… A method of influencing brain behaviour (the 'neuro' part of the phrase) through the use of language (the 'linguistic' part) and other types of communication to enable a person to 'recode' the way the brain responds to stimuli (that's the 'programming') and manifest new and better behaviours. Neuro-Linguistic Programming often incorporates hypnosis and self-hypnosis to help achieve the change (or 'programming') that is wanted.

British alternative media operation UKColumn has done very detailed research into Common Purpose over a long period. I quoted co-founder and former naval officer Brian Gerrish in my book *Remember Who You Are*, published in 2011, as saying the following years before current times:

It is interesting that many of the mothers who have had children taken by the State speak of the Social Services people being icily cool, emotionless and, as two ladies said in slightly different words, '… like little robots'. We know that NLP is cumulative, so people can be given small imperceptible doses of NLP in a course here, another in a few months, next year etc. In this way, major changes are accrued in their personality, but the day by day change is almost unnoticeable.

In these and other ways 'graduates' have had their perceptions uniformly reframed and they return to their roles in the institutions of government, law enforcement, legal profession, military, 'education', the UK National Health Service and the whole swathe of the establishment structure to pursue a common agenda preparing for the 'post-industrial', 'post-democratic' society. I say 'preparing' but we are now there. 'Post-industrial' is code for the Great Reset and 'post-democratic' is 'Covid' fascism. UKColumn has spoken to partners of those who have attended Common Purpose 'training'. They have described how personalities and attitudes of 'graduates' changed very noticeably for the worse by the time they had completed the course. They had been 'reframed' and told they are the 'leaders' – the special ones – who know better than the population. There has also been the very demonstrable recruitment of psychopaths and narcissists into government administration at all

levels and law enforcement. If you want psychopathy hire psychopaths and you get a simple cause and effect. If you want administrators, police officers and 'leaders' to perceive the public as lesser beings who don't matter then employ narcissists. These personalities are identified using 'psychometrics' that identifies knowledge, abilities, attitudes and personality traits, mostly through carefully-designed questionnaires and tests. As this policy has passed through the decades we have had power-crazy, power-trippers appointed into law enforcement, security and government administration in preparation for current times and the dynamic between public and law enforcement/officialdom has been transformed. UKColumn's Brian Gerrish said of the narcissistic personality:

> Their love of themselves and power automatically means that they will crush others who get in their way. I received a major piece of the puzzle when a friend pointed out that when they made public officials re-apply for their own jobs several years ago they were also required to do psychometric tests. This was undoubtedly the start of the screening process to get 'their' sort of people in post.

How obvious that has been since 2020 although it was clear what was happening long before if people paid attention to the changing public-establishment dynamic.

Change agents

At the centre of events in 'Covid' Britain is the National Health Service (NHS) which has behaved disgracefully in slavishly following the Cult agenda. The NHS management structure is awash with Common Purpose graduates or 'change agents' working to a common cause. Helen Bevan, a Chief of Service Transformation at the NHS Institute for Innovation and Improvement, co-authored a document called 'Towards a million change agents, a review of the social movements literature: implications for large scale change in the NHS'. The document compared a project management approach to that of change and social movements where 'people change

themselves and each other – peer to peer'. Two definitions given for a 'social movement' were:

A group of people who consciously attempt to build a radically new social order; involves people of a broad range of social backgrounds; and deploys politically confrontational and socially disruptive tactics – Cyrus Zirakzadeh 1997

Collective challenges, based on common purposes and social solidarities, in sustained interaction with elites, opponents, and authorities – Sidney Tarrow 1994

Helen Bevan wrote another NHS document in which she defined 'framing' as 'the process by which leaders construct, articulate and put across their message in a powerful and compelling way in order to win people to their cause and call them to action'. I think I could come up with another definition that would be rather more accurate. The National Health Service and institutions of Britain and the wider world have been taken over by reframed 'change agents' and that includes everything from the United Nations to national governments, local councils and social services which have been kidnapping children from loving parents on an extraordinary and gathering scale on the road to the end of parenthood altogether. Children from loving homes are stolen and kidnapped by the state and put into the 'care' (inversion) of the local authority through council homes, foster parents and forced adoption. At the same time children are allowed to be abused without response while many are under council 'care'. UKColumn highlighted the Common Purpose connection between South Yorkshire Police and Rotherham council officers in the case of the scandal in that area of the sexual exploitation of children to which the authorities turned not one blind eye, but both:

We were alarmed to discover that the Chief Executive, the Strategic Director of Children and Young People's Services, the Manager for the Local Strategic Partnership, the Community Cohesion Manager, the Cabinet Member for Cohesion, the Chief Constable and his predecessor had all attended Leadership training courses provided by the pseudo-charity Common Purpose.

Once 'change agents' have secured positions of hire and fire within any organisation things start to move very quickly. Personnel are then hired and fired on the basis of whether they will work towards the agenda the change agent represents. If they do they are rapidly promoted even though they may be incompetent. Those more qualified and skilled who are pre-Common Purpose 'old school' see their careers stall and even disappear. This has been happening for decades in every institution of state, police, 'health' and social services and all of them have been transformed as a result in their attitudes to their jobs and the public. Medical professions, including nursing, which were once vocations for the caring now employ many cold, callous and couldn't give a shit personality types. The UKColumn investigation concluded:

By blurring the boundaries between people, professions, public and private sectors, responsibility and accountability, Common Purpose encourages 'graduates' to believe that as new selected leaders, they can work together, outside of the established political and social structures, to achieve a paradigm shift or CHANGE – so called 'Leading Beyond Authority'. In doing so, the allegiance of the individual becomes 'reframed' on CP colleagues and their NETWORK.

Reframing the Face-Nappies

Nowhere has this process been more obvious than in the police where recruitment of psychopaths and development of unquestioning mind-controlled group-thinkers have transformed law enforcement into a politically-correct 'Woke' joke and a travesty of what should be public service. Today they wear their face-nappies like good little gofers and enforce 'Covid' rules which are fascism under another name. Alongside the specifically-recruited psychopaths we have software minds incapable of free thought. Brian Gerrish again:

An example is the policeman who would not get on a bike for a press photo because he had not done the cycling proficiency course. Normal people say this is political correctness gone mad. Nothing could be further from the truth. The policeman has been reframed, and in his reality it is perfect common sense not to get on the bike 'because he hasn't done the cycling course'.

Another example of this is where the police would not rescue a boy from a pond until they had taken advice from above on the 'risk assessment'. A normal person would have arrived, perhaps thought of the risk for a moment, and dived in. To the police now 'reframed', they followed 'normal' procedure.

There are shocking cases of reframed ambulance crews doing the same. Sheer unthinking stupidity of London Face-Nappies headed by Common Purpose graduate Cressida Dick can be seen in their behaviour at a vigil in March, 2021, for a murdered woman, Sarah Everard. A police officer had been charged with the crime. Anyone with a brain would have left the vigil alone in the circumstances. Instead they 'manhandled' women to stop them breaking 'Covid rules' to betray classic reframing. Minds in the thrall of perception control have no capacity for seeing a situation on its merits and acting accordingly. 'Rules is rules' is their only mind-set. My father used to say that rules and regulations are for the guidance of the intelligent and the blind obedience of the idiot. Most of the intelligent, decent, coppers have gone leaving only the other kind and a few old school for whom the job must be a daily nightmare. The combination of psychopaths and rule-book software minds has been clearly on public display in the 'Covid' era with automaton robots in uniform imposing fascistic 'Covid' regulations on the population without any personal initiative or judging situations on their merits. There are thousands of examples around the world, but I'll make my point with the infamous Derbyshire police in the English East Midlands – the ones who think pouring dye into beauty spots and using drones to track people walking in the countryside away from anyone is called 'policing'. To them there are rules decreed by the government which they have to enforce and in their bewildered state a group gathering in a closed space and someone walking alone in the countryside are the same thing. It is beyond idiocy and enters the realm of clinical insanity.

Police officers in Derbyshire said they were 'horrified' – *horrified* – to find 15 to 20 'irresponsible' kids playing a football match at a closed leisure centre 'in breach of coronavirus restrictions'. When they saw the police the kids ran away leaving their belongings behind and the reframed men and women of Derbyshire police were seeking to establish their identities with a view to fining their parents. The most natural thing for youngsters to do – kicking a ball about – is turned into a criminal activity and enforced by the moronic software programs of Derbyshire police. You find the same mentality in every country. These barely conscious 'horrified' officers said they had to take action because 'we need to ensure these rules are being followed' and 'it is of the utmost importance that you ensure your children are following the rules and regulations for Covid-19'. Had any of them done ten seconds of research to see if this parroting of their masters' script could be supported by any evidence? Nope. Reframed people don't think – others think for them and that's the whole idea of reframing. I have seen police officers one after the other repeating without question word for word what officialdom tells them just as I have seen great swathes of the public doing the same. Ask either for 'their' opinion and out spews what they have been told to think by the official narrative. Police and public may seem to be in different groups, but their mentality is the same. Most people do whatever they are told in fear not doing so or because they believe what officialdom tells them; almost the entirety of the police do what they are told for the same reason. Ultimately it's the tiny inner core of the global Cult that's telling both what to do.

So Derbyshire police were 'horrified'. Oh, really? Why did they think those kids were playing football? It was to relieve the psychological consequences of lockdown and being denied human contact with their friends and interaction, touch and discourse vital to human psychological health. Being denied this month after month has dismantled the psyche of many children and young people as depression and suicide have exploded. Were Derbyshire police *horrified by that*? Are you kidding? Reframed people don't have those

mental and emotional processes that can see how the impact on the psychological health of youngsters is far more dangerous than any 'virus' even if you take the mendacious official figures to be true. The reframed are told (programmed) how to act and so they do. The Derbyshire Chief Constable in the first period of lockdown when the black dye and drones nonsense was going on was Peter Goodman. He was the man who severed the connection between his force and the Derbyshire Constabulary *Male Voice* Choir when he decided that it was not inclusive enough to allow women to join. The fact it was a male voice choir making a particular sound produced by male voices seemed to elude a guy who terrifyingly ran policing in Derbyshire. He retired weeks after his force was condemned as disgraceful by former Supreme Court Justice Jonathan Sumption for their behaviour over extreme lockdown impositions. Goodman was replaced by his deputy Rachel Swann who was in charge when her officers were 'horrified'. The police statement over the boys committing the hanging-offence of playing football included the line about the youngsters being 'irresponsible in the times we are all living through' missing the point that the real relevance of the 'times we are all living through' is the imposition of fascism enforced by psychopaths and reframed minds of police officers playing such a vital part in establishing the fascist tyranny that their own children and grandchildren will have to live in their entire lives. As a definition of insanity that is hard to beat although it might be run close by imposing masks on people that can have a serious effect on their health while wearing a face nappy all day themselves. Once again public and police do it for the same reason – the authorities tell them to and who are they to have the self-respect to say no?

Wokers in uniform

How reframed do you have to be to arrest a *six-year-old* and take him to court for *picking a flower* while waiting for a bus? Brain dead police and officialdom did just that in North Carolina where criminal proceedings happen regularly for children under nine. Attorney Julie Boyer gave the six-year-old crayons and a colouring book

during the 'flower' hearing while the 'adults' decided his fate. County Chief District Court Judge Jay Corpening asked: 'Should a child that believes in Santa Claus, the Easter Bunny and the tooth fairy be making life-altering decisions?' Well, of course not, but common sense has no meaning when you have a common purpose and a reframed mind. Treating children in this way, and police operating in American schools, is all part of the psychological preparation for children to accept a police state as normal all their adult lives. The same goes for all the cameras and biometric tracking technology in schools. Police training is focused on reframing them as snowflake Wokers and this is happening in the military. Pentagon top brass said that 'training sessions on extremism' were needed for troops who asked why they were so focused on the Capitol Building riot when Black Lives Matter riots were ignored. What's the difference between them some apparently and rightly asked. Actually, there is a difference. Five people died in the Capitol riot, only one through violence, and that was a police officer shooting an unarmed protestor. BLM riots killed at least 25 people and cost billions. Asking the question prompted the psychopaths and reframed minds that run the Pentagon to say that more 'education' (programming) was needed. Troop training is all based on psychological programming to make them fodder for the Cult – 'Military men are just dumb, stupid animals to be used as pawns in foreign policy' as Cult-to-his-DNA former Secretary of State Henry Kissinger famously said. Governments see the police in similar terms and it's time for those among them who can see this to defend the people and stop being enforcers of the Cult agenda upon the people.

The US military, like the country itself, is being targeted for destruction through a long list of Woke impositions. Cult-owned gaga 'President' Biden signed an executive order when he took office to allow taxpayer money to pay for transgender surgery for active military personnel and veterans. Are you a man soldier? No, I'm a LGBTQIA+ with a hint of Skoliosexual and Spectrasexual. Oh, good man. Bad choice of words you bigot. The Pentagon announced in March, 2021, the appointment of the first 'diversity and inclusion

officer' for US Special Forces. Richard Torres-Estrada arrived with the publication of a 'D&I Strategic Plan which will guide the enterprise-wide effort to institutionalize and sustain D&I'. If you think a Special Forces 'Strategic Plan' should have something to do with defending America you haven't been paying attention. Defending Woke is now the military's new role. Torres-Estrada has posted images comparing Donald Trump with Adolf Hitler and we can expect no bias from him as a representative of the supposedly non-political Pentagon. Cable news host Tucker Carlson said: 'The Pentagon is now the Yale faculty lounge but with cruise missiles.' Meanwhile Secretary of Defense Lloyd Austin, a board member of weapons-maker Raytheon with stock and compensation interests in October, 2020, worth $1.4 million, said he was purging the military of the 'enemy within' – anyone who isn't Woke and supports Donald Trump. Austin refers to his targets as 'racist extremists' while in true Woke fashion being himself a racist extremist. Pentagon documents pledge to 'eradicate, eliminate and conquer all forms of racism, sexism and homophobia'. The definitions of these are decided by 'diversity and inclusion committees' peopled by those who see racism, sexism and homophobia in every situation and opinion. Woke (the Cult) is dismantling the US military and purging testosterone as China expands its military and gives its troops 'masculinity training'. How do we think that is going to end when this is all Cult coordinated? The US military, like the British military, is controlled by Woke and spineless top brass who just go along with it out of personal career interests.

'Woke' means fast asleep

Mind control and perception manipulation techniques used on individuals to create group-think have been unleashed on the global population in general. As a result many have no capacity to see the obvious fascist agenda being installed all around them or what 'Covid' is really all about. Their brains are firewalled like a computer system not to process certain concepts, thoughts and realisations that are bad for the Cult. The young are most targeted as the adults they

will be when the whole fascist global state is planned to be fully implemented. They need to be prepared for total compliance to eliminate all pushback from entire generations. The Cult has been pouring billions into taking complete control of 'education' from schools to universities via its operatives and corporations and not least Bill Gates as always. The plan has been to transform 'education' institutions into programming centres for the mentality of 'Woke'. James McConnell, professor of psychology at the University of Michigan, wrote in *Psychology Today* in 1970:

> The day has come when we can combine sensory deprivation with drugs, hypnosis, and astute manipulation of reward and punishment, to gain almost absolute control over an individual's behaviour. It should then be possible to achieve a very rapid and highly effective type of brainwashing that would allow us to make dramatic changes in a person's behaviour and personality ...
>
> … We should reshape society so that we all would be trained from birth to want to do what society wants us to do. We have the techniques to do it... no-one owns his own personality you acquired, and there's no reason to believe you should have the right to refuse to acquire a new personality if your old one is anti-social.

This was the potential for mass brainwashing in 1970 and the mentality there displayed captures the arrogant psychopathy that drives it forward. I emphasise that not all young people have succumbed to Woke programming and those that haven't are incredibly impressive people given that today's young are the most perceptually-targeted generations in history with all the technology now involved. Vast swathes of the young generations, however, have fallen into the spell – and that's what it is – of Woke. The Woke mentality and perceptual program is founded on *inversion* and you will appreciate later why that is so significant. Everything with Woke is inverted and the opposite of what it is claimed to be. Woke was a term used in African-American culture from the 1900s and referred to an awareness of social and racial justice. This is not the meaning of the modern version or 'New Woke' as I call it in *The Answer*. Oh, no, Woke today means something very different no matter how much Wokers may seek to hide that and insist Old Woke and New

Woke are the same. See if you find any 'awareness of social justice' here in the modern variety:

- Woke demands 'inclusivity' while excluding anyone with a different opinion and calls for mass censorship to silence other views.

- Woke claims to stand against oppression when imposing oppression is the foundation of all that it does. It is the driver of political correctness which is nothing more than a Cult invention to manipulate the population to silence itself.

- Woke believes itself to be 'liberal' while pursuing a global society that can only be described as fascist (see 'anti-fascist' fascist Antifa).

- Woke calls for 'social justice' while spreading injustice wherever it goes against the common 'enemy' which can be easily identified as a differing view.

- Woke is supposed to be a metaphor for 'awake' when it is solid-gold asleep and deep in a Cult-induced coma that meets the criteria for 'off with the fairies'.

I state these points as obvious facts if people only care to look. I don't do this with a sense of condemnation. We need to appreciate that the onslaught of perceptual programming on the young has been incessant and merciless. I can understand why so many have been reframed, or, given their youth, framed from the start to see the world as the Cult demands. The Cult has had access to their minds day after day in its 'education' system for their entire formative years. Perception is formed from information received and the Cult-created system is a life-long download of information delivered to elicit a particular perception, thus behaviour. The more this has expanded into still new extremes in recent decades and ever-increasing censorship has deleted other opinions and information why wouldn't that lead to a perceptual reframing on a mass scale? I

have described already cradle-to-grave programming and in more recent times the targeting of young minds from birth to adulthood has entered the stratosphere. This has taken the form of skewing what is 'taught' to fit the Cult agenda and the omnipresent techniques of group-think to isolate non-believers and pressure them into line. There has always been a tendency to follow the herd, but we really are in a new world now in relation to that. We have parents who can see the 'Covid' hoax told by their children not to stop them wearing masks at school, being 'Covid' tested or having the 'vaccine' in fear of the peer-pressure consequences of being different. What is 'peer-pressure' if not pressure to conform to group-think? Renegade Minds never group-think and always retain a set of perceptions that are unique to them. Group-think is always underpinned by consequences for not group-thinking. Abuse now aimed at those refusing DNA-manipulating 'Covid vaccines' are a potent example of this. The biggest pressure to conform comes from the very group which is itself being manipulated. 'I am programmed to be part of a hive mind and so you must be.'

Woke control structures in 'education' now apply to every mainstream organisation. Those at the top of the 'education' hierarchy (the Cult) decide the policy. This is imposed on governments through the Cult network; governments impose it on schools, colleges and universities; their leadership impose the policy on teachers and academics and they impose it on children and students. At any level where there is resistance, perhaps from a teacher or university lecturer, they are targeted by the authorities and often fired. Students themselves regularly demand the dismissal of academics (increasingly few) at odds with the narrative that the students have been programmed to believe in. It is quite a thought that students who are being targeted by the Cult become so consumed by programmed group-think that they launch protests and demand the removal of those who are trying to push back against those targeting the students. Such is the scale of perceptual inversion. We see this with 'Covid' programming as the Cult imposes the rules via psycho-psychologists and governments on

shops, transport companies and businesses which impose them on their staff who impose them on their customers who pressure Pushbackers to conform to the will of the Cult which is in the process of destroying them and their families. Scan all aspects of society and you will see the same sequence every time.

Fact free Woke and hijacking the 'left'

There is no more potent example of this than 'Woke', a mentality only made possible by the deletion of factual evidence by an 'education' system seeking to produce an ever more uniform society. Why would you bother with facts when you don't know any? Deletion of credible history both in volume and type is highly relevant. Orwell said: 'Who controls the past controls the future: who controls the present controls the past.' They who control the perception of the past control the perception of the future and they who control the present control the perception of the past through the writing and deleting of history. Why would you oppose the imposition of Marxism in the name of Wokeism when you don't know that Marxism cost at least 100 million lives in the 20th century alone? Watch videos and read reports in which Woker generations are asked basic historical questions – it's mind-blowing. A survey of 2,000 people found that six percent of millennials (born approximately early1980s to early 2000s) believed the Second World War (1939-1945) broke out with the assassination of President Kennedy (in 1963) and one in ten thought Margaret Thatcher was British Prime Minister at the time. She was in office between 1979 and 1990. We are in a post-fact society. Provable facts are no defence against the fascism of political correctness or Silicon Valley censorship. Facts don't matter anymore as we have witnessed with the 'Covid' hoax. Sacrificing uniqueness to the Woke group-think religion is all you are required to do and that means thinking for yourself is the biggest Woke no, no. All religions are an expression of group-think and censorship and Woke is just another religion with an orthodoxy defended by group-think and censorship. Burned at

the stake becomes burned on Twitter which leads back eventually to burned at the stake as Woke humanity regresses to ages past.

The biggest Woke inversion of all is its creators and funders. I grew up in a traditional left of centre political household on a council estate in Leicester in the 1950s and 60s – you know, the left that challenged the power of wealth-hoarding elites and threats to freedom of speech and opinion. In those days students went on marches defending freedom of speech while today's Wokers march for its deletion. What on earth could have happened? Those very elites (collectively the Cult) that we opposed in my youth and early life have funded into existence the antithesis of that former left and hijacked the 'brand' while inverting everything it ever stood for. We have a mentality that calls itself 'liberal' and 'progressive' while acting like fascists. Cult billionaires and their corporations have funded themselves into control of 'education' to ensure that Woke programming is unceasing throughout the formative years of children and young people and that non-Wokers are isolated (that word again) whether they be students, teachers or college professors. The Cult has funded into existence the now colossal global network of Woke organisations that have spawned and promoted all the 'causes' on the Cult wish-list for global transformation and turned Wokers into demanders of them. Does anyone really think it's a coincidence that the Cult agenda for humanity is a carbon (sorry) copy of the societal transformations desired by Woke?? These are only some of them:

Political correctness: The means by which the Cult deletes all public debates that it knows it cannot win if we had the free-flow of information and evidence.

Human-caused 'climate change': The means by which the Cult seeks to transform society into a globally-controlled dictatorship imposing its will over the fine detail of everyone's lives 'to save the planet' which doesn't actually need saving.

Transgender obsession: Preparing collective perception to accept the 'new human' which would not have genders because it would be created technologically and not through procreation. I'll have much more on this in Human 2.0.

Race obsession: The means by which the Cult seeks to divide and rule the population by triggering racial division through the perception that society is more racist than ever when the opposite is the case. Is it perfect in that regard? No. But to compare today with the racism of apartheid and segregation brought to an end by the civil rights movement in the 1960s is to insult the memory of that movement and inspirations like Martin Luther King. Why is the 'anti-racism' industry (which it is) so dominated by privileged white people?

White supremacy: This is a label used by privileged white people to demonise poor and deprived white people pushing back on tyranny to marginalise and destroy them. White people are being especially targeted as the dominant race by number within Western society which the Cult seeks to transform in its image. If you want to change a society you must weaken and undermine its biggest group and once you have done that by using the other groups you next turn on them to do the same … 'Then they came for the Jews and I was not a Jew so I did nothing.'

Mass migration: The mass movement of people from the Middle East, Africa and Asia into Europe, from the south into the United States and from Asia into Australia are another way the Cult seeks to dilute the racial, cultural and political influence of white people on Western society. White people ask why their governments appear to be working against them while being politically and culturally biased towards incoming cultures. Well, here's your answer. In the same way sexually 'straight' people, men and women, ask why the

authorities are biased against them in favour of other sexualities. The answer is the same – that's the way the Cult wants it to be for very sinister motives.

These are all central parts of the Cult agenda and central parts of the Woke agenda and Woke was created and continues to be funded to an immense degree by Cult billionaires and corporations. If anyone begins to say 'coincidence' the syllables should stick in their throat.

Billionaire 'social justice warriors'

Joe Biden is a 100 percent-owned asset of the Cult and the Wokers' man in the White House whenever he can remember his name and for however long he lasts with his rapidly diminishing cognitive function. Even walking up the steps of an aircraft without falling on his arse would appear to be a challenge. He's not an empty-shell puppet or anything. From the minute Biden took office (or the Cult did) he began his executive orders promoting the Woke wish-list. You will see the Woke agenda imposed ever more severely because it's really the *Cult* agenda. Woke organisations and activist networks spawned by the Cult are funded to the extreme so long as they promote what the Cult wants to happen. Woke is funded to promote 'social justice' by billionaires who become billionaires by destroying social justice. The social justice mantra is only a cover for dismantling social justice and funded by billionaires that couldn't give a damn about social justice. Everything makes sense when you see that. One of Woke's premier funders is Cult billionaire financier George Soros who said: 'I am basically there to make money, I cannot and do not look at the social consequences of what I do.' This is the same Soros who has given more than $32 billion to his Open Society Foundations global Woke network and funded Black Lives Matter, mass immigration into Europe and the United States, transgender activism, climate change activism, political correctness and groups targeting 'white supremacy' in the form of privileged white thugs that dominate Antifa. What a scam it all is and when

you are dealing with the unquestioning fact-free zone of Woke scamming them is child's play. All you need to pull it off in all these organisations are a few in-the-know agents of the Cult and an army of naïve, reframed, uninformed, narcissistic, know-nothings convinced of their own self-righteousness, self-purity and virtue.

Soros and fellow billionaires and billionaire corporations have poured hundreds of millions into Black Lives Matter and connected groups and promoted them to a global audience. None of this is motivated by caring about black people. These are the billionaires that have controlled and exploited a system that leaves millions of black people in abject poverty and deprivation which they do absolutely nothing to address. The same Cult networks funding BLM were behind the *slave trade!* Black Lives Matter hijacked a phrase that few would challenge and they have turned this laudable concept into a political weapon to divide society. You know that BLM is a fraud when it claims that *All* Lives Matter, the most inclusive statement of all, is 'racist'. BLM and its Cult masters don't want to end racism. To them it's a means to an end to control all of humanity never mind the colour, creed, culture or background. What has destroying the nuclear family got to do with ending racism? Nothing – but that is one of the goals of BLM and also happens to be a goal of the Cult as I have been exposing in my books for decades. Stealing children from loving parents and giving schools ever more power to override parents is part of that same agenda. BLM is a Marxist organisation and why would that not be the case when the Cult created Marxism *and* BLM? Patrisse Cullors, a BLM co-founder, said in a 2015 video that she and her fellow organisers, including co-founder Alicia Garza, are 'trained Marxists'. The lady known after marriage as Patrisse Khan-Cullors bought a $1.4 million home in 2021 in one of the whitest areas of California with a black population of just 1.6 per cent and has so far bought *four* high-end homes for a total of $3.2 million. How very Marxist. There must be a bit of spare in the BLM coffers, however, when Cult corporations and billionaires have handed over the best part of $100 million. Many black people can see that Black Lives Matter is not

working for them, but against them, and this is still more confirmation. Black journalist Jason Whitlock, who had his account suspended by Twitter for simply linking to the story about the 'Marxist's' home buying spree, said that BLM leaders are 'making millions of dollars off the backs of these dead black men who they wouldn't spit on if they were on fire and alive'.

Black Lies Matter

Cult assets and agencies came together to promote BLM in the wake of the death of career criminal George Floyd who had been jailed a number of times including for forcing his way into the home of a black woman with others in a raid in which a gun was pointed at her stomach. Floyd was filmed being held in a Minneapolis street in 2020 with the knee of a police officer on his neck and he subsequently died. It was an appalling thing for the officer to do, but the same technique has been used by police on peaceful protestors of lockdown without any outcry from the Woke brigade. As unquestioning supporters of the Cult agenda Wokers have supported lockdown and all the 'Covid' claptrap while attacking anyone standing up to the tyranny imposed in its name. Court documents would later include details of an autopsy on Floyd by County Medical Examiner Dr Andrew Baker who concluded that Floyd had taken a fatal level of the drug fentanyl. None of this mattered to fact-free, question-free, Woke. Floyd's death was followed by worldwide protests against police brutality amid calls to defund the police. Throwing babies out with the bathwater is a Woke speciality. In the wake of the murder of British woman Sarah Everard a Green Party member of the House of Lords, Baroness Jones of Moulescoomb (Nincompoopia would have been better), called for a 6pm curfew for all men. This would be in breach of the Geneva Conventions on war crimes which ban collective punishment, but that would never have crossed the black and white Woke mind of Baroness Nincompoopia who would have been far too convinced of her own self-righteousness to compute such details. Many American cities did defund the police in the face of Floyd riots

and after $15 million was deleted from the police budget in Washington DC under useless Woke mayor Muriel Bowser car-jacking alone rose by 300 percent and within six months the US capital recorded its highest murder rate in 15 years. The same happened in Chicago and other cities in line with the Cult/Soros plan to bring fear to streets and neighbourhoods by reducing the police, releasing violent criminals and not prosecuting crime. This is the mob-rule agenda that I have warned in the books was coming for so long. Shootings in the area of Minneapolis where Floyd was arrested increased by 2,500 percent compared with the year before. Defunding the police over George Floyd has led to a big increase in dead people with many of them black. Police protection for politicians making these decisions stayed the same or increased as you would expect from professional hypocrites. The Cult doesn't actually want to abolish the police. It wants to abolish local control over the police and hand it to federal government as the psychopaths advance the Hunger Games Society. Many George Floyd protests turned into violent riots with black stores and businesses destroyed by fire and looting across America fuelled by Black Lives Matter. Woke doesn't do irony. If you want civil rights you must loot the liquor store and the supermarket and make off with a smart TV. It's the only way.

It's not a race war – it's a class war

Black people are patronised by privileged blacks and whites alike and told they are victims of white supremacy. I find it extraordinary to watch privileged blacks supporting the very system and bloodline networks behind the slave trade and parroting the same Cult-serving manipulative crap of their privileged white, often billionaire, associates. It is indeed not a race war but a class war and colour is just a diversion. Black Senator Cory Booker and black Congresswoman Maxine Waters, more residents of Nincompoopia, personify this. Once you tell people they are victims of someone else you devalue both their own responsibility for their plight and the power they have to impact on their reality and experience. Instead

we have: 'You are only in your situation because of whitey – turn on them and everything will change.' It won't change. Nothing changes in our lives unless *we* change it. Crucial to that is never seeing yourself as a victim and always as the creator of your reality. Life is a simple sequence of choice and consequence. Make different choices and you create different consequences. *You* have to make those choices – not Black Lives Matter, the Woke Mafia and anyone else that seeks to dictate your life. Who are they these Wokers, an emotional and psychological road traffic accident, to tell you what to do? Personal empowerment is the last thing the Cult and its Black Lives Matter want black people or anyone else to have. They claim to be defending the underdog while *creating* and perpetuating the underdog. The Cult's worst nightmare is human unity and if they are going to keep blacks, whites and every other race under economic servitude and control then the focus must be diverted from what they have in common to what they can be manipulated to believe divides them. Blacks have to be told that their poverty and plight is the fault of the white bloke living on the street in the same poverty and with the same plight they are experiencing. The difference is that your plight black people is due to him, a white supremacist with 'white privilege' living on the street. Don't unite as one human family against your mutual oppressors and suppressors – fight the oppressor with the white face who is as financially deprived as you are. The Cult knows that as its 'Covid' agenda moves into still new levels of extremism people are going to respond and it has been spreading the seeds of disunity everywhere to stop a united response to the evil that targets *all of us*.

Racist attacks on 'whiteness' are getting ever more outrageous and especially through the American Democratic Party which has an appalling history for anti-black racism. Barack Obama, Joe Biden, Hillary Clinton and Nancy Pelosi all eulogised about Senator Robert Byrd at his funeral in 2010 after a nearly 60-year career in Congress. Byrd was a brutal Ku Klux Klan racist and a violent abuser of Cathy O'Brien in MKUltra. He said he would never fight in the military 'with a negro by my side' and 'rather I should die a thousand times,

and see Old Glory trampled in the dirt never to rise again, than to see this beloved land of ours become degraded by race mongrels, a throwback to the blackest specimen from the wilds'. Biden called Byrd a 'very close friend and mentor'. These 'Woke' hypocrites are not anti-racist they are anti-poor and anti-people not of their perceived class. Here is an illustration of the scale of anti-white racism to which we have now descended. Seriously Woke and moronic *New York Times* contributor Damon Young described whiteness as a 'virus' that 'like other viruses will not die until there are no bodies left for it to infect'. He went on: '… the only way to stop it is to locate it, isolate it, extract it, and kill it.' Young can say that as a black man with no consequences when a white man saying the same in reverse would be facing a jail sentence. *That's* racism. We had super-Woke numbskull senators Tammy Duckworth and Mazie Hirono saying they would object to future Biden Cabinet appointments if he did not nominate more Asian Americans and Pacific Islanders. Never mind the ability of the candidate what do they look like? Duckworth said: 'I will vote for racial minorities and I will vote for LGBTQ, but anyone else I'm not voting for.' Appointing people on the grounds of race is illegal, but that was not a problem for this ludicrous pair. They were on-message and that's a free pass in any situation.

Critical race racism

White children are told at school they are intrinsically racist as they are taught the divisive 'critical race theory'. This claims that the law and legal institutions are inherently racist and that race is a socially constructed concept used by white people to further their economic and political interests at the expense of people of colour. White is a 'virus' as we've seen. Racial inequality results from 'social, economic, and legal differences that white people create between races to maintain white interests which leads to poverty and criminality in minority communities'. I must tell that to the white guy sleeping on the street. The principal of East Side Community School in New York sent white parents a manifesto that called on

them to become 'white traitors' and advocate for full 'white abolition'. These people are teaching your kids when they urgently need a psychiatrist. The 'school' included a chart with 'eight white identities' that ranged from 'white supremacist' to 'white abolition' and defined the behaviour white people must follow to end 'the regime of whiteness'. Woke blacks and their privileged white associates are acting exactly like the slave owners of old and Ku Klux Klan racists like Robert Byrd. They are too full of their own self-purity to see that, but it's true. Racism is not a body type; it's a state of mind that can manifest through any colour, creed or culture.

Another racial fraud is *'equity'*. Not equality of treatment and opportunity – equity. It's a term spun as equality when it means something very different. Equality in its true sense is a raising up while 'equity' is a race to the bottom. Everyone in the same level of poverty is 'equity'. Keep everyone down – that's equity. The Cult doesn't want anyone in the human family to be empowered and BLM leaders, like all these 'anti-racist' organisations, continue their privileged, pampered existence by perpetuating the perception of gathering racism. When is the last time you heard an 'anti-racist' or 'anti-Semitism' organisation say that acts of racism and discrimination have *fallen?* It's not in the interests of their fund-raising and power to influence and the same goes for the professional soccer anti-racism operation, Kick It Out. Two things confirmed that the Black Lives Matter riots in the summer of 2020 were Cult creations. One was that while anti-lockdown protests were condemned in this same period for 'transmitting 'Covid' the authorities supported mass gatherings of Black Lives Matter supporters. I even saw self-deluding people claiming to be doctors say the two types of protest were not the same. No – the non-existent 'Covid' was in favour of lockdowns and attacked those that protested against them while 'Covid' supported Black Lives Matter and kept well away from its protests. The whole thing was a joke and as lockdown protestors were arrested, often brutally, by reframed Face-Nappies we had the grotesque sight of police officers taking the knee to Black Lives Matter, a Cult-funded Marxist

organisation that supports violent riots and wants to destroy the nuclear family and white people.

He's not white? Shucks!

Woke obsession with race was on display again when ten people were shot dead in Boulder, Colorado, in March, 2021. Cult-owned Woke TV channels like CNN said the shooter appeared to be a white man and Wokers were on Twitter condemning 'violent white men' with the usual mantras. Then the shooter's name was released as Ahmad Al Aliwi Alissa, an anti-Trump Arab-American, and the sigh of disappointment could be heard five miles away. Never mind that ten people were dead and what that meant for their families. Race baiting was all that mattered to these sick Cult-serving people like Barack Obama who exploited the deaths to further divide America on racial grounds which is his job for the Cult. This is the man that 'racist' white Americans made the first black president of the United States and then gave him a second term. Not-very-bright Obama has become filthy rich on the back of that and today appears to have a big influence on the Biden administration. Even so he's still a downtrodden black man and a victim of white supremacy. This disingenuous fraud reveals the contempt he has for black people when he puts on a Deep South Alabama accent whenever he talks to them, no, *at* them.

Another BLM red flag was how the now fully-Woke (fully-Cult) and fully-virtue-signalled professional soccer authorities had their teams taking the knee before every match in support of Marxist Black Lives Matter. Soccer authorities and clubs displayed 'Black Lives Matter' on the players' shirts and flashed the name on electronic billboards around the pitch. Any fans that condemned what is a Freemasonic taking-the-knee ritual were widely condemned as you would expect from the Woke virtue-signallers of professional sport and the now fully-Woke media. We have reverse racism in which you are banned from criticising any race or culture except for white people for whom anything goes – say what you like, no problem. What has this got to do with racial harmony and

equality? We've had black supremacists from Black Lives Matter telling white people to fall to their knees in the street and apologise for their white supremacy. Black supremacists acting like white supremacist slave owners of the past couldn't breach their self-obsessed, race-obsessed sense of self-purity. Joe Biden appointed a race-obsessed black supremacist Kristen Clarke to head the Justice Department Civil Rights Division. Clarke claimed that blacks are endowed with 'greater mental, physical and spiritual abilities' than whites. If anyone reversed that statement they would be vilified. Clarke is on-message so no problem. She's never seen a black-white situation in which the black figure is anything but a virtuous victim and she heads the Civil Rights Division which should treat everyone the same or it isn't civil rights. Another perception of the Renegade Mind: If something or someone is part of the Cult agenda they will be supported by Woke governments and media no matter what. If they're not, they will be condemned and censored. It really is that simple and so racist Clarke prospers despite (make that because of) her racism.

The end of culture

Biden's administration is full of such racial, cultural and economic bias as the Cult requires the human family to be divided into warring factions. We are now seeing racially-segregated graduations and everything, but everything, is defined through the lens of perceived 'racism. We have 'racist' mathematics, 'racist' food and even 'racist' *plants*. World famous Kew Gardens in London said it was changing labels on plants and flowers to tell its pre-'Covid' more than two million visitors a year how racist they are. Kew director Richard Deverell said this was part of an effort to 'move quickly to decolonise collections' after they were approached by one Ajay Chhabra 'an actor with an insight into how sugar cane was linked to slavery'. They are *plants* you idiots. 'Decolonisation' in the Woke manual really means colonisation of society with its mentality and by extension colonisation by the Cult. We are witnessing a new Chinese-style 'Cultural Revolution' so essential to the success of all

Marxist takeovers. Our cultural past and traditions have to be swept away to allow a new culture to be built-back-better. Woke targeting of long-standing Western cultural pillars including historical monuments and cancelling of historical figures is what happened in the Mao revolution in China which 'purged remnants of capitalist and traditional elements from Chinese society' and installed Maoism as the dominant ideology'. For China see the Western world today and for 'dominant ideology' see Woke. Better still see Marxism or Maoism. The 'Covid' hoax has specifically sought to destroy the arts and all elements of Western culture from people meeting in a pub or restaurant to closing theatres, music venues, sports stadiums, places of worship and even banning *singing*. Destruction of Western society is also why criticism of any religion is banned except for Christianity which again is the dominant religion as white is the numerically-dominant race. Christianity may be fading rapidly, but its history and traditions are weaved through the fabric of Western society. Delete the pillars and other structures will follow until the whole thing collapses. I am not a Christian defending that religion when I say that. I have no religion. It's just a fact. To this end Christianity has itself been turned Woke to usher its own downfall and its ranks are awash with 'change agents' – knowing and unknowing – at every level including Pope Francis (*definitely* knowing) and the clueless Archbishop of Canterbury Justin Welby (possibly not, but who can be sure?). Woke seeks to coordinate attacks on Western culture, traditions, and ways of life through 'intersectionality' defined as 'the complex, cumulative way in which the effects of multiple forms of discrimination (such as racism, sexism, and classism) combine, overlap, or intersect especially in the experiences of marginalised individuals or groups'. Wade through the Orwellian Woke-speak and this means coordinating disparate groups in a common cause to overthrow freedom and liberal values.

The entire structure of public institutions has been infested with Woke – government at all levels, political parties, police, military, schools, universities, advertising, media and trade unions. This abomination has been achieved through the Cult web by appointing

Wokers to positions of power and battering non-Wokers into line through intimidation, isolation and threats to their job. Many have been fired in the wake of the empathy-deleted, vicious hostility of 'social justice' Wokers and the desire of gutless, spineless employers to virtue-signal their Wokeness. Corporations are filled with Wokers today, most notably those in Silicon Valley. Ironically at the top they are not Woke at all. They are only exploiting the mentality their Cult masters have created and funded to censor and enslave while the Wokers cheer them on until it's their turn. Thus the Woke 'liberal left' is an inversion of the traditional liberal left. Campaigning for justice on the grounds of power and wealth distribution has been replaced by campaigning for identity politics. The genuine traditional left would never have taken money from today's billionaire abusers of fairness and justice and nor would the billionaires have wanted to fund that genuine left. It would not have been in their interests to do so. The division of opinion in those days was between the haves and have nots. This all changed with Cult manipulated and funded identity politics. The division of opinion today is between Wokers and non-Wokers and not income brackets. Cult corporations and their billionaires may have taken wealth disparity to cataclysmic levels of injustice, but as long as they speak the language of Woke, hand out the dosh to the Woke network and censor the enemy they are 'one of us'. Billionaires who don't give a damn about injustice are laughing at them till their bellies hurt. Wokers are not even close to self-aware enough to see that. The transformed 'left' dynamic means that Wokers who drone on about 'social justice' are funded by billionaires that have destroyed social justice the world over. It's *why* they are billionaires.

The climate con

Nothing encapsulates what I have said more comprehensively than the hoax of human-caused global warming. I have detailed in my books over the years how Cult operatives and organisations were the pump-primers from the start of the climate con. A purpose-built vehicle for this is the Club of Rome established by the Cult in 1968

with the Rockefellers and Rothschilds centrally involved all along. Their gofer frontman Maurice Strong, a Canadian oil millionaire, hosted the Earth Summit in Rio de Janeiro, Brazil, in 1992 where the global 'green movement' really expanded in earnest under the guiding hand of the Cult. The Earth Summit established Agenda 21 through the Cult-created-and-owned United Nations to use the illusion of human-caused climate change to justify the transformation of global society to save the world from climate disaster. It is a No-Problem-Reaction-Solution sold through governments, media, schools and universities as whole generations have been terrified into believing that the world was going to end in their lifetimes unless what old people had inflicted upon them was stopped by a complete restructuring of how everything is done. Chill, kids, it's all a hoax. Such restructuring is precisely what the Cult agenda demands (purely by coincidence of course). Today this has been given the codename of the Great Reset which is only an updated term for Agenda 21 and its associated Agenda 2030. The latter, too, is administered through the UN and was voted into being by the General Assembly in 2015. Both 21 and 2030 seek centralised control of all resources and food right down to the raindrops falling on your own land. These are some of the demands of Agenda 21 established in 1992. See if you recognise this society emerging today:

- End national sovereignty
- State planning and management of all land resources, ecosystems, deserts, forests, mountains, oceans and fresh water; agriculture; rural development; biotechnology; and ensuring *'equity'*
- The state to 'define the role' of business and financial resources
- Abolition of private property
- 'Restructuring' the family unit (see BLM)
- Children raised by the state
- People told what their job will be
- Major restrictions on movement
- Creation of 'human settlement zones'

- Mass resettlement as people are forced to vacate land where they live
- Dumbing down education
- Mass global depopulation in pursuit of all the above

The United Nations was created as a Trojan horse for world government. With the climate con of critical importance to promoting that outcome you would expect the UN to be involved. Oh, it's involved all right. The UN is promoting Agenda 21 and Agenda 2030 justified by 'climate change' while also driving the climate hoax through its Intergovernmental Panel on Climate Change (IPCC), one of the world's most corrupt organisations. The IPCC has been lying ferociously and constantly since the day it opened its doors with the global media hanging unquestioningly on its every mendacious word. The Green movement is entirely Woke and has long lost its original environmental focus since it was co-opted by the Cult. An obsession with 'global warming' has deleted its values and scrambled its head. I experienced a small example of what I mean on a beautiful country walk that I have enjoyed several times a week for many years. The path merged into the fields and forests and you felt at one with the natural world. Then a 'Green' organisation, the Hampshire and Isle of Wight Wildlife Trust, took over part of the land and proceeded to cut down a large number of trees, including mature ones, to install a horrible big, bright steel 'this-is-ours-stay-out' fence that destroyed the whole atmosphere of this beautiful place. No one with a feel for nature would do that. Day after day I walked to the sound of chainsaws and a magnificent mature weeping willow tree that I so admired was cut down at the base of the trunk. When I challenged a Woke young girl in a green shirt (of course) about this vandalism she replied: 'It's a weeping willow – it will grow back.' This is what people are paying for when they donate to the Hampshire and Isle of Wight Wildlife Trust and many other 'green' organisations today. It is not the environmental movement that I knew and instead has become a support-system – as with Extinction Rebellion – for a very dark agenda.

Private jets for climate justice

The Cult-owned, Gates-funded, World Economic Forum and its founder Klaus Schwab were behind the emergence of Greta Thunberg to harness the young behind the climate agenda and she was invited to speak to the world at … the UN. Schwab published a book, *Covid-19: The Great Reset* in 2020 in which he used the 'Covid' hoax and the climate hoax to lay out a new society straight out of Agenda 21 and Agenda 2030. Bill Gates followed in early 2021 when he took time out from destroying the world to produce a book in his name about the way to save it. Gates flies across the world in private jets and admitted that 'I probably have one of the highest greenhouse gas footprints of anyone on the planet … my personal flying alone is gigantic.' He has also bid for the planet's biggest private jet operator. Other climate change saviours who fly in private jets include John Kerry, the US Special Presidential Envoy for Climate, and actor Leonardo DiCaprio, a 'UN Messenger of Peace with special focus on climate change'. These people are so full of bullshit they could corner the market in manure. We mustn't be sceptical, though, because the Gates book, *How to Avoid a Climate Disaster: The Solutions We Have and the Breakthroughs We Need*, is a genuine attempt to protect the world and not an obvious pile of excrement attributed to a mega-psychopath aimed at selling his masters' plans for humanity. The Gates book and the other shite-pile by Klaus Schwab could have been written by the same person and may well have been. Both use 'climate change' and 'Covid' as the excuses for their new society and by coincidence the Cult's World Economic Forum and Bill and Melinda Gates Foundation promote the climate hoax and hosted Event 201 which pre-empted with a 'simulation' the very 'coronavirus' hoax that would be simulated for real on humanity within weeks. The British 'royal' family is promoting the 'Reset' as you would expect through Prince 'climate change caused the war in Syria' Charles and his hapless son Prince William who said that we must 'reset our relationship with nature and our trajectory as a species' to avoid a climate disaster. Amazing how many promotors of the 'Covid' and 'climate change' control

systems are connected to Gates and the World Economic Forum. A 'study' in early 2021 claimed that carbon dioxide emissions must fall by the equivalent of a global lockdown roughly every two years for the next decade to save the planet. The 'study' appeared in the same period that the Schwab mob claimed in a video that lockdowns destroying the lives of billions are good because they make the earth 'quieter' with less 'ambient noise'. They took down the video amid a public backlash for such arrogant, empathy-deleted stupidity You see, however, where they are going with this. Corinne Le Quéré, a professor at the Tyndall Centre for Climate Change Research, University of East Anglia, was lead author of the climate lockdown study, and she writes for … the World Economic Forum. Gates calls in 'his' book for changing 'every aspect of the economy' (long-time Cult agenda) and for humans to eat synthetic 'meat' (predicted in my books) while cows and other farm animals are eliminated. Australian TV host and commentator Alan Jones described what carbon emission targets would mean for farm animals in Australia alone if emissions were reduced as demanded by 35 percent by 2030 and zero by 2050:

Well, let's take agriculture, the total emissions from agriculture are about 75 million tonnes of carbon dioxide, equivalent. Now reduce that by 35 percent and you have to come down to 50 million tonnes, I've done the maths. So if you take for example 1.5 million cows, you're going to have to reduce the herd by 525,000 [by] 2030, nine years, that's 58,000 cows a year. The beef herd's 30 million, reduce that by 35 percent, that's 10.5 million, which means 1.2 million cattle have to go every year between now and 2030. This is insanity!

There are 75 million sheep. Reduce that by 35 percent, that's 26 million sheep, that's almost 3 million a year. So under the Paris Agreement over 30 million beasts. dairy cows, cattle, pigs and sheep would go. More than 8,000 every minute of every hour for the next decade, do these people know what they're talking about?

Clearly they don't at the level of campaigners, politicians and administrators. The Cult *does* know; that's the outcome it wants. We are faced with not just a war on humanity. Animals and the natural world are being targeted and I have been saying since the 'Covid' hoax began that the plan eventually was to claim that the 'deadly virus' is able to jump from animals, including farm animals and

domestic pets, to humans. Just before this book went into production came this story: 'Russia registers world's first Covid-19 vaccine for cats & dogs as makers of Sputnik V warn pets & farm animals could spread virus'. The report said 'top scientists warned that the deadly pathogen could soon begin spreading through homes and farms' and 'the next stage is the infection of farm and domestic animals'. Know the outcome and you'll see the journey. Think what that would mean for animals and keep your eye on a term called zoonosis or zoonotic diseases which transmit between animals and humans. The Cult wants to break the connection between animals and people as it does between people and people. Farm animals fit with the Cult agenda to transform food from natural to synthetic.

The gas of life is killing us

There can be few greater examples of Cult inversion than the condemnation of carbon dioxide as a dangerous pollutant when it is the gas of life. Without it the natural world would be dead and so we would all be dead. We breathe in oxygen and breathe out carbon dioxide while plants produce oxygen and absorb carbon dioxide. It is a perfect symbiotic relationship that the Cult wants to dismantle for reasons I will come to in the final two chapters. Gates, Schwab, other Cult operatives and mindless repeaters, want the world to be 'carbon neutral' by at least 2050 and the earlier the better. 'Zero carbon' is the cry echoed by lunatics calling for 'Zero Covid' when we already have it. These carbon emission targets will deindustrialise the world in accordance with Cult plans – the post-industrial, post-democratic society – and with so-called renewables like solar and wind not coming even close to meeting human energy needs blackouts and cold are inevitable. Texans got the picture in the winter of 2021 when a snow storm stopped wind turbines and solar panels from working and the lights went down along with water which relies on electricity for its supply system. Gates wants everything to be powered by electricity to ensure that his masters have the kill switch to stop all human activity, movement, cooking, water and warmth any time they like. The climate lie is so

stupendously inverted that it claims we must urgently reduce carbon dioxide when we *don't have enough*.

Co2 in the atmosphere is a little above 400 parts per million when the optimum for plant growth is 2,000 ppm and when it falls anywhere near 150 ppm the natural world starts to die and so do we. It fell to as low as 280 ppm in an 1880 measurement in Hawaii and rose to 413 ppm in 2019 with industrialisation which is why the planet has become *greener* in the industrial period. How insane then that psychopathic madman Gates is not satisfied only with blocking the rise of Co2. He's funding technology to suck it out of the atmosphere. The reason why will become clear. The industrial era is not destroying the world through Co2 and has instead turned around a potentially disastrous ongoing fall in Co2. Greenpeace co-founder and scientist Patrick Moore walked away from Greenpeace in 1986 and has exposed the green movement for fear-mongering and lies. He said that 500 million years ago there was *17 times* more Co2 in the atmosphere than we have today and levels have been falling for hundreds of millions of years. In the last 150 million years Co2 levels in Earth's atmosphere had reduced by *90 percent*. Moore said that by the time humanity began to unlock carbon dioxide from fossil fuels we were at '38 seconds to midnight' and in that sense: 'Humans are [the Earth's] salvation.' Moore made the point that only half the Co2 emitted by fossil fuels stays in the atmosphere and we should remember that all pollution pouring from chimneys that we are told is carbon dioxide is in fact nothing of the kind. It's pollution. Carbon dioxide is an invisible gas.

William Happer, Professor of Physics at Princeton University and long-time government adviser on climate, has emphasised the Co2 deficiency for maximum growth and food production. Greenhouse growers don't add carbon dioxide for a bit of fun. He said that most of the warming in the last 100 years, after the earth emerged from the super-cold period of the 'Little Ice Age' into a natural warming cycle, was over by 1940. Happer said that a peak year for warming in 1988 can be explained by a 'monster El Nino' which is a natural and cyclical warming of the Pacific that has nothing to do with 'climate

change'. He said the effect of Co2 could be compared to painting a wall with red paint in that once two or three coats have been applied it didn't matter how much more you slapped on because the wall will not get much redder. Almost all the effect of the rise in Co2 has already happened, he said, and the volume in the atmosphere would now have to *double* to increase temperature by a single degree. Climate hoaxers know this and they have invented the most ridiculously complicated series of 'feedback' loops to try to overcome this rather devastating fact. You hear puppet Greta going on cluelessly about feedback loops and this is why.

The Sun affects temperature? No you *climate denier*

Some other nonsense to contemplate: Climate graphs show that rises in temperature do not follow rises in Co2 – *it's the other way round* with a lag between the two of some 800 years. If we go back 800 years from present time we hit the Medieval Warm Period when temperatures were higher than now without any industrialisation and this was followed by the Little Ice Age when temperatures plummeted. The world was still emerging from these centuries of serious cold when many climate records began which makes the ever-repeated line of the 'hottest year since records began' meaningless when you are not comparing like with like. The coldest period of the Little Ice Age corresponded with the lowest period of sunspot activity when the Sun was at its least active. Proper scientists will not be at all surprised by this when it confirms the obvious fact that earth temperature is affected by the scale of Sun activity and the energetic power that it subsequently emits; but when is the last time you heard a climate hoaxer talking about the Sun as a source of earth temperature?? Everything has to be focussed on Co2 which makes up just 0.117 percent of so-called greenhouse gases and only a fraction of even that is generated by human activity. The rest is natural. More than *90 percent* of those greenhouse gases are water vapour and clouds (Fig 9). Ban moisture I say. Have you noticed that the climate hoaxers no longer use the polar bear as their promotion image? That's because far from becoming extinct polar

bear communities are stable or thriving. Joe Bastardi, American meteorologist, weather forecaster and outspoken critic of the climate lie, documents in his book *The Climate Chronicles* how weather patterns and events claimed to be evidence of climate change have been happening since long before industrialisation: 'What happened before naturally is happening again, as is to be expected given the cyclical nature of the climate due to the design of the planet.' If you read the detailed background to the climate hoax in my other books you will shake your head and wonder how anyone could believe the crap which has spawned a multi-trillion dollar industry based on absolute garbage (see HIV causes AIDs and Sars-Cov-2 causes 'Covid-19'). Climate and 'Covid' have much in common given they have the same source. They both have the contradictory *everything* factor in which everything is explained by reference to them. It's hot – 'it's climate change'. It's cold – 'it's climate change'. I got a sniffle – 'it's Covid'. I haven't got a sniffle – 'it's Covid'. Not having a sniffle has to be a symptom of 'Covid'. Everything is and not having a sniffle is especially dangerous if you are a slow walker. For sheer audacity I offer you a Cambridge University 'study' that actually linked 'Covid' to 'climate change'. It had to happen eventually. They concluded that climate change played a role in 'Covid-19' spreading from animals to humans because … wait for it … I kid you not … *the two groups were forced closer together as populations grow*. Er, that's it. The whole foundation on which this depended was that 'Bats are the likely zoonotic origin of SARS-CoV-1 and SARS-CoV-2'. Well, they are not. They are nothing to do with it. Apart from bats not being the origin and therefore 'climate change' effects on bats being irrelevant I am in awe of their academic insight. Where would we be without them? Not where we are that's for sure.

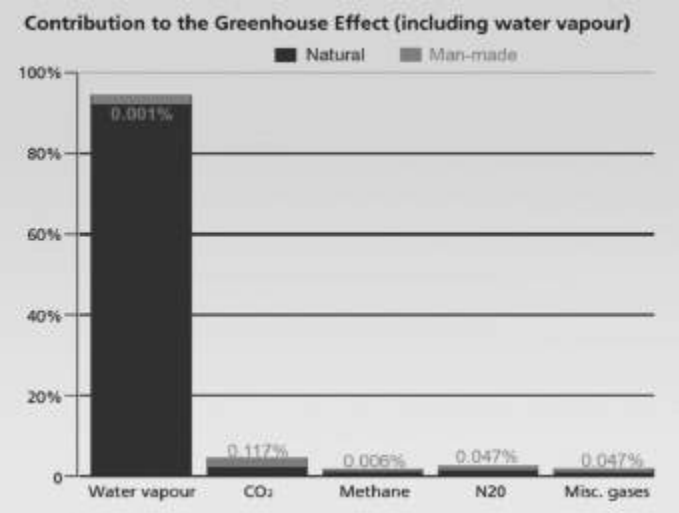

Figure 9: The idea that the gas of life is disastrously changing the climate is an insult to brain cell activity.

One other point about the weather is that climate modification is now well advanced and not every major weather event is natural – or earthquake come to that. I cover this subject at some length in other books. China is openly planning a rapid expansion of its weather modification programme which includes changing the climate in an area more than one and a half times the size of India. China used weather manipulation to ensure clear skies during the 2008 Olympics in Beijing. I have quoted from US military documents detailing how to employ weather manipulation as a weapon of war and they did that in the 1960s and 70s during the conflict in Vietnam with Operation Popeye manipulating monsoon rains for military purposes. Why would there be international treaties on weather modification if it wasn't possible? Of course it is. Weather is energetic information and it can be changed.

How was the climate hoax pulled off? See 'Covid'

If you can get billions to believe in a 'virus' that doesn't exist you can get them to believe in human-caused climate change that doesn't exist. Both are being used by the Cult to transform global society in the way it has long planned. Both hoaxes have been achieved in pretty much the same way. First you declare a lie is a fact. There's a

'virus' you call SARS-Cov-2 or humans are warming the planet with their behaviour. Next this becomes, via Cult networks, the foundation of government, academic and science policy and belief. Those who parrot the mantra are given big grants to produce research that confirms the narrative is true and ever more 'symptoms' are added to make the 'virus'/'climate change' sound even more scary. Scientists and researchers who challenge the narrative have their grants withdrawn and their careers destroyed. The media promote the lie as the unquestionable truth and censor those with an alternative view or evidence. A great percentage of the population believe what they are told as the lie becomes an everybody-knows-that and the believing-masses turn on those with a mind of their own. The technique has been used endlessly throughout human history. Wokers are the biggest promotors of the climate lie *and* 'Covid' fascism because their minds are owned by the Cult; their sense of self-righteous self-purity knows no bounds; and they exist in a bubble of reality in which facts are irrelevant and only get in the way of looking without seeing.

Running through all of this like veins in a blue cheese is control of information, which means control of perception, which means control of behaviour, which collectively means control of human society. The Cult owns the global media and Silicon Valley fascists for the simple reason that it *has* to. Without control of information it can't control perception and through that human society. Examine every facet of the Cult agenda and you will see that anything supporting its introduction is never censored while anything pushing back is always censored. I say again: Psychopaths that know why they are doing this must go before Nuremberg trials and those that follow their orders must trot along behind them into the same dock. 'I was just following orders' didn't work the first time and it must not work now. Nuremberg trials must be held all over the world before public juries for politicians, government officials, police, compliant doctors, scientists and virologists, and all Cult operatives such as Gates, Tedros, Fauci, Vallance, Whitty, Ferguson, Zuckerberg, Wojcicki, Brin, Page, Dorsey, the whole damn lot of

them – including, no *especially*, the psychopath psychologists. Without them and the brainless, gutless excuses for journalists that have repeated their lies, none of this could be happening. Nobody can be allowed to escape justice for the psychological and economic Armageddon they are all responsible for visiting upon the human race.

As for the compliant, unquestioning, swathes of humanity, and the self-obsessed, all-knowing ignorance of the Wokers … don't start me. God help their kids. God help their grandkids. God *help them*.

CHAPTER NINE

We must have it? So what is it?

Well I won't back down. No, I won't back down. You can stand me up at the Gates of Hell. But I won't back down
Tom Petty

I will now focus on the genetically-manipulating 'Covid vaccines' which do not meet this official definition of a vaccine by the US Centers for Disease Control (CDC): 'A product that stimulates a person's immune system to produce immunity to a specific disease, protecting the person from that disease.' On that basis 'Covid vaccines' are not a vaccine in that the makers don't even claim they stop infection or transmission.

They are instead part of a multi-levelled conspiracy to change the nature of the human body and what it means to be 'human' and to depopulate an enormous swathe of humanity. What I shall call Human 1.0 is on the cusp of becoming Human 2.0 and for very sinister reasons. Before I get to the 'Covid vaccine' in detail here's some background to vaccines in general. Government regulators do not test vaccines – the makers do – and the makers control which data is revealed and which isn't. Children in America are given 50 vaccine doses by age six and 69 by age 19 and the effect of the whole combined schedule has never been tested. Autoimmune diseases when the immune system attacks its own body have soared in the mass vaccine era and so has disease in general in children and the young. Why wouldn't this be the case when vaccines target the *immune system?* The US government gave Big Pharma drug

companies immunity from prosecution for vaccine death and injury in the 1986 National Childhood Vaccine Injury Act (NCVIA) and since then the government (taxpayer) has been funding compensation for the consequences of Big Pharma vaccines. The criminal and satanic drug giants can't lose and the vaccine schedule has increased dramatically since 1986 for this reason. There is no incentive to make vaccines safe and a big incentive to make money by introducing ever more. Even against a ridiculously high bar to prove vaccine liability, and with the government controlling the hearing in which it is being challenged for compensation, the vaccine court has so far paid out more than $4 billion. These are the vaccines we are told are safe and psychopaths like Zuckerberg censor posts saying otherwise. The immunity law was even justified by a ruling that vaccines by their nature were 'unavoidably unsafe'.

Check out the ingredients of vaccines and you will be shocked if you are new to this. *They put that in children's bodies?? What??* Try aluminium, a brain toxin connected to dementia, aborted foetal tissue and formaldehyde which is used to embalm corpses. World-renowned aluminium expert Christopher Exley had his research into the health effect of aluminium in vaccines shut down by Keele University in the UK when it began taking funding from the Bill and Melinda Gates Foundation. Research when diseases 'eradicated' by vaccines began to decline and you will find the fall began long *before* the vaccine was introduced. Sometimes the fall even plateaued after the vaccine. Diseases like scarlet fever for which there was no vaccine declined in the same way because of environmental and other factors. A perfect case in point is the polio vaccine. Polio began when lead arsenate was first sprayed as an insecticide and residues remained in food products. Spraying started in 1892 and the first US polio epidemic came in Vermont in 1894. The simple answer was to stop spraying, but Rockefeller-created Big Pharma had a better idea. Polio was decreed to be caused by the *poliovirus* which 'spreads from person to person and can infect a person's spinal cord'. Lead arsenate was replaced by the lethal DDT which had the same effect of causing paralysis by damaging the brain and central nervous

system. Polio plummeted when DDT was reduced and then banned, but the vaccine is still given the credit for something it didn't do. Today by far the biggest cause of polio is the vaccines promoted by Bill Gates. Vaccine justice campaigner Robert Kennedy Jr, son of assassinated (by the Cult) US Attorney General Robert Kennedy, wrote:

> In 2017, the World Health Organization (WHO) reluctantly admitted that the global explosion in polio is predominantly vaccine strain. The most frightening epidemics in Congo, Afghanistan, and the Philippines, are all linked to vaccines. In fact, by 2018, 70% of global polio cases were vaccine strain.

Vaccines make fortunes for Cult-owned Gates and Big Pharma while undermining the health and immune systems of the population. We had a glimpse of the mentality behind the Big Pharma cartel with a report on WION (World is One News), an international English language TV station based in India, which exposed the extraordinary behaviour of US drug company Pfizer over its 'Covid vaccine'. The WION report told how Pfizer had made fantastic demands of Argentina, Brazil and other countries in return for its 'vaccine'. These included immunity from prosecution, even for Pfizer negligence, government insurance to protect Pfizer from law suits and handing over as collateral sovereign assets of the country to include Argentina's bank reserves, military bases and embassy buildings. Pfizer demanded the same of Brazil in the form of waiving sovereignty of its assets abroad; exempting Pfizer from Brazilian laws; and giving Pfizer immunity from all civil liability. This is a 'vaccine' developed with government funding. Big Pharma is evil incarnate as a creation of the Cult and all must be handed tickets to Nuremberg.

Phantom 'vaccine' for a phantom 'disease'

I'll expose the 'Covid vaccine' fraud and then go on to the wider background of why the Cult has set out to 'vaccinate' every man, woman and child on the planet for an alleged 'new disease' with a survival rate of 99.77 percent (or more) even by the grotesquely-

manipulated figures of the World Health Organization and Johns Hopkins University. The 'infection' to 'death' ratio is 0.23 to 0.15 percent according to Stanford epidemiologist Dr John Ioannidis and while estimates vary the danger remains tiny. I say that if the truth be told the fake infection to fake death ratio is zero. Never mind all the evidence I have presented here and in *The Answer* that there is no 'virus' let us just focus for a moment on that death-rate figure of say 0.23 percent. The figure includes all those worldwide who have tested positive with a test not testing for the 'virus' and then died within 28 days or even longer of any other cause – *any other cause*. Now subtract all those illusory 'Covid' deaths on the global data sheets from the 0.23 percent. What do you think you would be left with? *Zero*. A vaccination has never been successfully developed for a so-called coronavirus. They have all failed at the animal testing stage when they caused hypersensitivity to what they were claiming to protect against and made the impact of a disease far worse. Cult-owned vaccine corporations got around that problem this time by bypassing animal trials, going straight to humans and making the length of the 'trials' before the public rollout as short as they could get away with. Normally it takes five to ten years or more to develop vaccines that still cause demonstrable harm to many people and that's without including the long-term effects that are never officially connected to the vaccination. 'Covid' non-vaccines have been officially produced and approved in a matter of months from a standing start and part of the reason is that (a) they were developed before the 'Covid' hoax began and (b) they are based on computer programs and not natural sources. Official non-trials were so short that government agencies gave *emergency*, not full, approval. 'Trials' were not even completed and full approval cannot be secured until they are. Public 'Covid vaccination' is actually a *continuation of the trial*. Drug company 'trials' are not scheduled to end until 2023 by which time a lot of people are going to be dead. Data on which government agencies gave this emergency approval was supplied by the Big Pharma corporations themselves in the form of Pfizer/BioNTech, AstraZeneca, Moderna, Johnson & Johnson, and

others, and this is the case with all vaccines. By its very nature *emergency* approval means drug companies do not have to prove that the 'vaccine' is 'safe and effective'. How could they with trials way short of complete? Government regulators only have to *believe* that they *could* be safe and effective. It is criminal manipulation to get products in circulation with no testing worth the name. Agencies giving that approval are infested with Big Pharma-connected place-people and they act in the interests of Big Pharma (the Cult) and not the public about whom they do not give a damn.

More human lab rats

'Covid vaccines' produced in record time by Pfizer/BioNTech and Moderna employ a technique *never approved before for use on humans*. They are known as mRNA 'vaccines' and inject a synthetic version of 'viral' mRNA or 'messenger RNA'. The key is in the term 'messenger'. The body works, or doesn't, on the basis of information messaging. Communications are constantly passing between and within the genetic system and the brain. Change those messages and you change the state of the body and even its very nature and you can change psychology and behaviour by the way the brain processes information. I think you are going to see significant changes in personality and perception of many people who have had the 'Covid vaccine' synthetic potions. Insider Aldous Huxley predicted the following in 1961 and mRNA 'vaccines' can be included in the term 'pharmacological methods':

> There will be, in the next generation or so, a pharmacological method of making people love their servitude, and producing dictatorship without tears, so to speak, producing a kind of painless concentration camp for entire societies, so that people will in fact have their own liberties taken away from them, but rather enjoy it, because they will be distracted from any desire to rebel by propaganda or brainwashing, or brainwashing enhanced by pharmacological methods. And this seems to be the final revolution.

Apologists claim that mRNA synthetic 'vaccines' don't change the DNA genetic blueprint because RNA does not affect DNA only the other way round. This is so disingenuous. A process called 'reverse

transcription' can convert RNA into DNA and be integrated into DNA in the cell nucleus. This was highlighted in December, 2020, by scientists at Harvard and Massachusetts Institute of Technology (MIT). Geneticists report that more than 40 percent of mammalian genomes results from reverse transcription. On the most basic level if messaging changes then that sequence must lead to changes in DNA which is receiving and transmitting those communications. How can introducing synthetic material into cells not change the cells where DNA is located? The process is known as transfection which is defined as 'a technique to insert foreign nucleic acid (DNA or RNA) into a cell, typically with the intention of altering the properties of the cell'. Researchers at the Sloan Kettering Institute in New York found that changes in messenger RNA can deactivate tumour-suppressing proteins and thereby promote cancer. This is what happens when you mess with messaging. 'Covid vaccine' maker Moderna was founded in 2010 by Canadian stem cell biologist Derrick J. Rossi after his breakthrough discovery in the field of transforming and reprogramming stem cells. These are neutral cells that can be programmed to become any cell including sperm cells. Moderna was therefore founded on the principle of genetic manipulation and has never produced any vaccine or drug before its genetically-manipulating synthetic 'Covid' shite. Look at the name – Mode-RNA or Modify-RNA. Another important point is that the US Supreme Court has ruled that genetically-modified DNA, or complementary DNA (cDNA) synthesized in the laboratory from messenger RNA, can be patented and owned. These psychopaths are doing this to the human body.

Cells replicate synthetic mRNA in the 'Covid vaccines' and in theory the body is tricked into making antigens which trigger antibodies to target the 'virus spike proteins' which as Dr Tom Cowan said have *never been seen*. Cut the crap and these 'vaccines' deliver *self-replicating* synthetic material to the cells with the effect of changing human DNA. The more of them you have the more that process is compounded while synthetic material is all the time self-replicating. 'Vaccine'-maker Moderna describes mRNA as 'like

software for the cell' and so they are messing with the body's software. What happens when you change the software in a computer? Everything changes. For this reason the Cult is preparing a production line of mRNA 'Covid vaccines' and a long list of excuses to use them as with all the 'variants' of a 'virus' never shown to exist. The plan is further to transfer the mRNA technique to other vaccines mostly given to children and young people. The cumulative consequences will be a transformation of human DNA through a constant infusion of synthetic genetic material which will kill many and change the rest. Now consider that governments that have given emergency approval for a vaccine that's not a vaccine; never been approved for humans before; had no testing worth the name; and the makers have been given immunity from prosecution for any deaths or adverse effects suffered by the public. The UK government awarded *permanent legal indemnity* to itself and its employees for harm done when a patient is being treated for 'Covid-19' or 'suspected Covid-19'. That is quite a thought when these are possible 'side-effects' from the 'vaccine' (they are not 'side', they are effects) listed by the US Food and Drug Administration:

Guillain-Barre syndrome; acute disseminated encephalomyelitis; transverse myelitis; encephalitis; myelitis; encephalomyelitis; meningoencephalitis; meningitis; encephalopathy; convulsions; seizures; stroke; narcolepsy; cataplexy; anaphylaxis; acute myocardial infarction (heart attack); myocarditis; pericarditis; autoimmune disease; death; implications for pregnancy, and birth outcomes; other acute demyelinating diseases; non anaphylactic allergy reactions; thrombocytopenia ; disseminated intravascular coagulation; venous thromboembolism; arthritis; arthralgia; joint pain; Kawasaki disease; multisystem inflammatory syndrome in children; vaccine enhanced disease. The latter is the way the 'vaccine' has the potential to make diseases far worse than they would otherwise be.

UK doctor and freedom campaigner Vernon Coleman described the conditions in this list as 'all unpleasant, most of them very serious, and you can't get more serious than death'. The thought that anyone at all has had the 'vaccine' in these circumstances is testament to the potential that humanity has for clueless, unquestioning, stupidity and for many that programmed stupidity has already been terminal.

An insider speaks

Dr Michael Yeadon is a former Vice President, head of research and Chief Scientific Adviser at vaccine giant Pfizer. Yeadon worked on the inside of Big Pharma, but that did not stop him becoming a vocal critic of 'Covid vaccines' and their potential for multiple harms, including infertility in women. By the spring of 2021 he went much further and even used the no, no, term 'conspiracy'. When you begin to see what is going on it is impossible not to do so. Yeadon spoke out in an interview with freedom campaigner James Delingpole and I mentioned earlier how he said that no one had samples of 'the virus'. He explained that the mRNA technique originated in the anti-cancer field and ways to turn on and off certain genes which could be advantageous if you wanted to stop cancer growing out of control. 'That's the origin of them. They are a very unusual application, really.' Yeadon said that treating a cancer patient with an aggressive procedure might be understandable if the alternative was dying, but it was quite another thing to use the same technique as a public health measure. Most people involved wouldn't catch the infectious agent you were vaccinating against and if they did they probably wouldn't die:

> If you are really using it as a public health measure you really want to as close as you can get to zero sides-effects … I find it odd that they chose techniques that were really cutting their teeth in the field of oncology and I'm worried that in using gene-based vaccines that have to be injected in the body and spread around the body, get taken up into some cells, and the regulators haven't quite told us which cells they get taken up into … you are going to be generating a wide range of responses … with multiple steps each of which could go well or badly.

I doubt the Cult intends it to go well. Yeadon said that you can put any gene you like into the body through the 'vaccine'. 'You can certainly give them a gene that would do them some harm if you wanted.' I was intrigued when he said that when used in the cancer field the technique could turn genes on and off. I explore this process in *The Answer* and with different genes having different functions you could create mayhem – physically and psychologically – if you turned the wrong ones on and the right ones off. I read reports of an experiment by researchers at the University of Washington's school of computer science and engineering in which they encoded DNA to infect computers. The body is itself a biological computer and if human DNA can inflict damage on a computer why can't the computer via synthetic material mess with the human body? It can. The Washington research team said it was possible to insert malicious malware into 'physical DNA strands' and corrupt the computer system of a gene sequencing machine as it 'reads gene letters and stores them as binary digits 0 and 1'. They concluded that hackers could one day use blood or spit samples to access computer systems and obtain sensitive data from police forensics labs or infect genome files. It is at this level of digital interaction that synthetic 'vaccines' need to be seen to get the full picture and that will become very clear later on. Michael Yeadon said it made no sense to give the 'vaccine' to younger people who were in no danger from the 'virus'. What was the benefit? It was all downside with potential effects:

> The fact that my government in what I thought was a civilised, rational country, is raining [the 'vaccine'] on people in their 30s and 40s, even my children in their 20s, they're getting letters and phone calls, I know this is not right and any of you doctors who are vaccinating you know it's not right, too. They are not at risk. They are not at risk from the disease, so you are now hoping that the side-effects are so rare that you get away with it. You don't give new technology … that you don't understand to 100 percent of the population.

Blood clot problems with the AstraZeneca 'vaccine' have been affecting younger people to emphasise the downside risks with no benefit. AstraZeneca's version, produced with Oxford University, does not use mRNA, but still gets its toxic cocktail inside cells where

it targets DNA. The Johnson & Johnson 'vaccine' which uses a similar technique has also produced blood clot effects to such an extent that the United States paused its use at one point. They are all 'gene therapy' (cell modification) procedures and not 'vaccines'. The truth is that once the content of these injections enter cells we have no idea what the effect will be. People can speculate and some can give very educated opinions and that's good. In the end, though, only the makers know what their potions are designed to do and even they won't know every last consequence. Michael Yeadon was scathing about doctors doing what they knew to be wrong. 'Everyone's mute', he said. Doctors in the NHS must know this was not right, coming into work and injecting people. 'I don't know how they sleep at night. I know I couldn't do it. I know that if I were in that position I'd have to quit.' He said he knew enough about toxicology to know this was not a good risk-benefit. Yeadon had spoken to seven or eight university professors and all except two would not speak out publicly. Their universities had a policy that no one said anything that countered the government and its medical advisors. They were afraid of losing their government grants. This is how intimidation has been used to silence the truth at every level of the system. I say silence, but these people could still speak out if they made that choice. Yeadon called them 'moral cowards' – 'This is about your children and grandchildren's lives and you have just buggered off and left it.'

'Variant' nonsense

Some of his most powerful comments related to the alleged 'variants' being used to instil more fear, justify more lockdowns, and introduce more 'vaccines'. He said government claims about 'variants' were nonsense. He had checked the alleged variant 'codes' and they were 99.7 percent identical to the 'original'. This was the human identity difference equivalent to putting a baseball cap on and off or wearing it the other way round. A 0.3 percent difference would make it impossible for that 'variant' to escape immunity from the 'original'. This made no sense of having new 'vaccines' for

'variants'. He said there would have to be at least a *30 percent* difference for that to be justified and even then he believed the immune system would still recognise what it was. Gates-funded 'variant modeller' and 'vaccine'-pusher John Edmunds might care to comment. Yeadon said drug companies were making new versions of the 'vaccine' as a 'top up' for 'variants'. Worse than that, he said, the 'regulators' around the world like the MHRA in the UK had got together and agreed that because 'vaccines' for 'variants' were so similar to the first 'vaccines' *they did not have to do safety studies*. How transparently sinister that is. This is when Yeadon said: 'There is a conspiracy here.' There was no need for another vaccine for 'variants' and yet we were told that there was and the country had shut its borders because of them. 'They are going into hundreds of millions of arms without passing 'go' or any regulator. Why did they do that? Why did they pick this method of making the vaccine?'

The reason had to be something bigger than that it seemed and 'it's not protection against the virus'. It's was a far bigger project that meant politicians and advisers were willing to do things and not do things that knowingly resulted in avoidable deaths – 'that's already happened when you think about lockdown and deprivation of health care for a year.' He spoke of people prepared to do something that results in the avoidable death of their fellow human beings and it not bother them. This is the penny-drop I have been working to get across for more than 30 years – the level of pure evil we are dealing with. Yeadon said his friends and associates could not believe there could be that much evil, but he reminded them of Stalin, Pol Pot and Hitler and of what Stalin had said: 'One death is a tragedy. A million? A statistic.' He could not think of a benign explanation for why you need top-up vaccines 'which I'm sure you don't' and for the regulators 'to just get out of the way and wave them through'. Why would the regulators do that when they were still wrestling with the dangers of the 'parent' vaccine? He was clearly shocked by what he had seen since the 'Covid' hoax began and now he was thinking the previously unthinkable:

If you wanted to depopulate a significant proportion of the world and to do it in a way that doesn't involve destruction of the environment with nuclear weapons, poisoning everyone with anthrax or something like that, and you wanted plausible deniability while you had a multi-year infectious disease crisis, I actually don't think you could come up with a better plan of work than seems to be in front of me. I can't say that's what they are going to do, but I can't think of a benign explanation why they are doing it.

He said he never thought that they would get rid of 99 percent of humans, but now he wondered. 'If you wanted to that this would be a hell of a way to do it – it would be unstoppable folks.' Yeadon had concluded that those who submitted to the 'vaccine' would be allowed to have some kind of normal life (but for how long?) while screws were tightened to coerce and mandate the last few percent. 'I think they'll put the rest of them in a prison camp. I wish I was wrong, but I don't think I am.' Other points he made included: There were no coronavirus vaccines then suddenly they all come along at the same time; we have no idea of the long term affect with trials so short; coercing or forcing people to have medical procedures is against the Nuremberg Code instigated when the Nazis did just that; people should at least delay having the 'vaccine'; a quick Internet search confirms that masks don't reduce respiratory viral transmission and 'the government knows that'; they have smashed civil society and they know that, too; two dozen peer-reviewed studies show no connection between lockdown and reducing deaths; he knew from personal friends the elite were still flying around and going on holiday while the public were locked down; the elite were not having the 'vaccines'. He was also asked if 'vaccines' could be made to target difference races. He said he didn't know, but the document by the Project for the New American Century in September, 2000, said developing 'advanced forms of biological warfare that can target *specific genotypes* may transform biological warfare from the realm of terror to a politically useful tool.' Oh, they're evil all right. Of that we can be *absolutely* sure.

Another cull of old people

We have seen from the CDC definition that the mRNA 'Covid vaccine' is not a vaccine and nor are the others that *claim* to reduce 'severity of symptoms' in *some* people, but not protect from infection or transmission. What about all the lies about returning to 'normal' if people were 'vaccinated'? If they are not claimed to stop infection and transmission of the alleged 'virus', how does anything change? This was all lies to manipulate people to take the jabs and we are seeing that now with masks and distancing still required for the 'vaccinated'. How did they think that elderly people with fragile health and immune responses were going to be affected by infusing their cells with synthetic material and other toxic substances? They *knew* that in the short and long term it would be devastating and fatal as the culling of the old that began with the first lockdowns was continued with the 'vaccine'. Death rates in care homes soared immediately residents began to be 'vaccinated' – infused with synthetic material. Brave and committed whistleblower nurses put their careers at risk by exposing this truth while the rest kept their heads down and their mouths shut to put their careers before those they are supposed to care for. A long-time American Certified Nursing Assistant who gave his name as James posted a video in which he described emotionally what happened in his care home when vaccination began. He said that during 2020 very few residents were sick with 'Covid' and no one died during the entire year; but shortly after the Pfizer mRNA injections 14 people died within two weeks and many others were near death. 'They're dropping like flies', he said. Residents who walked on their own before the shot could no longer and they had lost their ability to conduct an intelligent conversation. The home's management said the sudden deaths were caused by a 'super-spreader' of 'Covid-19'. Then how come, James asked, that residents who refused to take the injections were not sick? It was a case of inject the elderly with mRNA synthetic potions and blame their illness and death that followed on the 'virus'. James described what was happening in care homes as 'the greatest crime of genocide this country has ever seen'. Remember the NHS staff nurse from earlier who used the same

word ‘genocide’ for what was happening with the ‘vaccines’ and that it was an ‘act of human annihilation’. A UK care home whistleblower told a similar story to James about the effect of the ‘vaccine’ in deaths and ‘outbreaks’ of illness dubbed ‘Covid’ after getting the jab. She told how her care home management and staff had zealously imposed government regulations and no one was allowed to even question the official narrative let alone speak out against it. She said the NHS was even worse. Again we see the results of reframing. A worker at a local care home where I live said they had not had a single case of ‘Covid’ there for almost a year and when the residents were ‘vaccinated’ they had 19 positive cases in two weeks with eight dying.

It’s not the ‘vaccine’ – honest

The obvious cause and effect was being ignored by the media and most of the public. Australia’s health minister Greg Hunt (a former head of strategy at the World Economic Forum) was admitted to hospital after he had the ‘vaccine’. He was suffering according to reports from the skin infection ‘cellulitis’ and it must have been a severe case to have warranted days in hospital. Immediately the authorities said this was nothing to do with the ‘vaccine’ when an effect of some vaccines is a ‘cellulitis-like reaction’. We had families of perfectly healthy old people who died after the ‘vaccine’ saying that if only they had been given the ‘vaccine’ earlier they would still be alive. As a numbskull rating that is off the chart. A father of four ‘died of Covid’ at aged 48 when he was taken ill two days after having the ‘vaccine’. The man, a health administrator, had been ‘shielding during the pandemic’ and had ‘not really left the house’ until he went for the ‘vaccine’. Having the ‘vaccine’ and then falling ill and dying does not seem to have qualified as a possible cause and effect and ‘Covid-19’ went on his death certificate. His family said they had no idea how he ‘caught the virus’. A family member said: ‘Tragically, it could be that going for a vaccination ultimately led to him catching Covid …The sad truth is that they are never going to know where it came from.’ The family warned people to remember

that the virus still existed and was 'very real'. So was their stupidity. Nurses and doctors who had the first round of the 'vaccine' were collapsing, dying and ending up in a hospital bed while they or their grieving relatives were saying they'd still have the 'vaccine' again despite what happened. I kid you not. You mean if your husband returned from the dead he'd have the same 'vaccine' again that killed him??

Doctors at the VCU Medical Center in Richmond, Virginia, said the Johnson & Johnson 'vaccine' was to blame for a man's skin peeling off. Patient Richard Terrell said: 'It all just happened so fast. My skin peeled off. It's still coming off on my hands now.' He said it was stinging, burning and itching and when he bent his arms and legs it was very painful with 'the skin swollen and rubbing against itself'. Pfizer/BioNTech and Moderna vaccines use mRNA to change the cell while the Johnson & Johnson version uses DNA in a process similar to AstraZeneca's technique. Johnson & Johnson and AstraZeneca have both had their 'vaccines' paused by many countries after causing serious blood problems. Terrell's doctor Fnu Nutan said he could have died if he hadn't got medical attention. It sounds terrible so what did Nutan and Terrell say about the 'vaccine' now? Oh, they still recommend that people have it. A nurse in a hospital bed 40 minutes after the vaccination and unable to swallow due to throat swelling was told by a doctor that he lost mobility in his arm for 36 hours following the vaccination. What did he say to the ailing nurse? 'Good for you for getting the vaccination.' We are dealing with a serious form of cognitive dissonance madness in both public and medical staff. There is a remarkable correlation between those having the 'vaccine' and trumpeting the fact and suffering bad happenings shortly afterwards. Witold Rogiewicz, a Polish doctor, made a video of his 'vaccination' and ridiculed those who were questioning its safety and the intentions of Bill Gates: 'Vaccinate yourself to protect yourself, your loved ones, friends and also patients. And to mention quickly I have info for anti-vaxxers and anti-Coviders if you want to contact Bill Gates you can do this through me.' He further ridiculed the dangers of 5G. Days later he

was dead, but naturally the vaccination wasn't mentioned in the verdict of 'heart attack'.

Lies, lies and more lies

So many members of the human race have slipped into extreme states of insanity and unfortunately they include reframed doctors and nursing staff. Having a 'vaccine' and dying within minutes or hours is not considered a valid connection while death from any cause within 28 days or longer of a positive test with a test not testing for the 'virus' means 'Covid-19' goes on the death certificate. How could that 'vaccine'-death connection not have been made except by calculated deceit? US figures in the initial rollout period to February 12th, 2020, revealed that a third of the deaths reported to the CDC after 'Covid vaccines' happened within 48 hours. Five men in the UK suffered an 'extremely rare' blood clot problem after having the AstraZeneca 'vaccine', but no causal link was established said the Gates-funded Medicines and Healthcare products Regulatory Agency (MHRA) which had given the 'vaccine' emergency approval to be used. Former Pfizer executive Dr Michael Yeadon explained in his interview how the procedures could cause blood coagulation and clots. People who should have been at no risk were dying from blood clots in the brain and he said he had heard from medical doctor friends that people were suffering from skin bleeding and massive headaches. The AstraZeneca 'shot' was stopped by some 20 countries over the blood clotting issue and still the corrupt MHRA, the European Medicines Agency (EMA) and the World Health Organization said that it should continue to be given even though the EMA admitted that it 'still cannot rule out definitively' a link between blood clotting and the 'vaccine'. Later Marco Cavaleri, head of EMA vaccine strategy, said there was indeed a clear link between the 'vaccine' and thrombosis, but they didn't know why. So much for the trials showing the 'vaccine' is safe. Blood clots were affecting younger people who would be under virtually no danger from 'Covid' even if it existed which makes it all the more stupid and sinister.

The British government responded to public alarm by wheeling out June Raine, the terrifyingly weak infant school headmistress sound-alike who heads the UK MHRA drug 'regulator'. The idea that she would stand up to Big Pharma and government pressure is laughable and she told us that all was well in the same way that she did when allowing untested, never-used-on-humans-before, genetically-manipulating 'vaccines' to be exposed to the public in the first place. Mass lying is the new normal of the 'Covid' era. The MHRA later said 30 cases of rare blood clots had by then been connected with the AstraZeneca 'vaccine' (that means a lot more in reality) while stressing that the benefits of the jab in preventing 'Covid-19' outweighed any risks. A more ridiculous and disingenuous statement with callous disregard for human health it is hard to contemplate. Immediately after the mendacious 'all-clears' two hospital workers in Denmark experienced blood clots and cerebral haemorrhaging following the AstraZeneca jab and one died. Top Norwegian health official Pål Andre Holme said the 'vaccine' was the only common factor: 'There is nothing in the patient history of these individuals that can give such a powerful immune response … I am confident that the antibodies that we have found are the cause, and I see no other explanation than it being the vaccine which triggers it.' Strokes, a clot or bleed in the brain, were clearly associated with the 'vaccine' from word of mouth and whistleblower reports. Similar consequences followed with all these 'vaccines' that we were told were so safe and as the numbers grew by the day it was clear we were witnessing human carnage.

Learning the hard way

A woman interviewed by UKColumn told how her husband suffered dramatic health effects after the vaccine when he'd been in good health all his life. He went from being a little unwell to losing all feeling in his legs and experiencing 'excruciating pain'. Misdiagnosis followed twice at Accident and Emergency (an 'allergy' and 'sciatica') before he was admitted to a neurology ward where doctors said his serious condition had been caused by the

'vaccine'. Another seven 'vaccinated' people were apparently being treated on the same ward for similar symptoms. The woman said he had the 'vaccine' because they believed media claims that it was safe. 'I didn't think the government would give out a vaccine that does this to somebody; I believed they would be bringing out a vaccination that would be safe.' What a tragic way to learn that lesson. Another woman posted that her husband was transporting stroke patients to hospital on almost every shift and when he asked them if they had been 'vaccinated' for 'Covid' they all replied 'yes'. One had a 'massive brain bleed' the day after his second dose. She said her husband reported the 'just been vaccinated' information every time to doctors in A and E only for them to ignore it, make no notes and appear annoyed that it was even mentioned. This particular report cannot be verified, but it expresses a common theme that confirms the monumental underreporting of 'vaccine' consequences. Interestingly as the 'vaccines' and their brain blood clot/stroke consequences began to emerge the UK National Health Service began a publicity campaign telling the public what to do in the event of a stroke. A Scottish NHS staff nurse who quit in disgust in March, 2021, said:

I have seen traumatic injuries from the vaccine, they're not getting reported to the yellow card [adverse reaction] scheme, they're treating the symptoms, not asking why, why it's happening. It's just treating the symptoms and when you speak about it you're dismissed like you're crazy, I'm not crazy, I'm not crazy because every other colleague I've spoken to is terrified to speak out, they've had enough.

Videos appeared on the Internet of people uncontrollably shaking after the 'vaccine' with no control over muscles, limbs and even their face. A Scottish mother broke out in a severe rash all over her body almost immediately after she was given the AstraZeneca 'vaccine'. The pictures were horrific. Leigh King, a 41-year-old hairdresser from Lanarkshire said: 'Never in my life was I prepared for what I was about to experience … My skin was so sore and constantly hot … I have never felt pain like this …' But don't you worry, the 'vaccine' is perfectly safe. Then there has been the effect on medical

staff who have been pressured to have the 'vaccine' by psychopathic 'health' authorities and government. A London hospital consultant who gave the name K. Polyakova wrote this to the *British Medical Journal* or *BMJ*:

> I am currently struggling with … the failure to report the reality of the morbidity caused by our current vaccination program within the health service and staff population. The levels of sickness after vaccination is unprecedented and staff are getting very sick and some with neurological symptoms which is having a huge impact on the health service function. Even the young and healthy are off for days, some for weeks, and some requiring medical treatment. Whole teams are being taken out as they went to get vaccinated together.
>
> Mandatory vaccination in this instance is stupid, unethical and irresponsible when it comes to protecting our staff and public health. We are in the voluntary phase of vaccination, and encouraging staff to take an unlicensed product that is impacting on their immediate health … it is clearly stated that these vaccine products do not offer immunity or stop transmission. In which case why are we doing it?

Not to protect health that's for sure. Medical workers are lauded by governments for agenda reasons when they couldn't give a toss about them any more than they can for the population in general. Schools across America faced the same situation as they closed due to the high number of teachers and other staff with bad reactions to the Pfizer/BioNTech, Moderna, and Johnson & Johnson 'Covid vaccines' all of which were linked to death and serious adverse effects. The *BMJ* took down the consultant's comments pretty quickly on the grounds that they were being used to spread 'disinformation'. They were exposing the truth about the 'vaccine' was the real reason. The cover-up is breathtaking.

Hiding the evidence

The scale of the 'vaccine' death cover-up worldwide can be confirmed by comparing official figures with the personal experience of the public. I heard of many people in my community who died immediately or soon after the vaccine that would never appear in the media or even likely on the official totals of 'vaccine' fatalities and adverse reactions when only about ten percent are estimated to be

reported and I have seen some estimates as low as one percent in a Harvard study. In the UK alone by April 29th, 2021, some 757,654 adverse reactions had been officially reported from the Pfizer/BioNTech, Oxford/AstraZeneca and Moderna 'vaccines' with more than a thousand deaths linked to jabs and that means an estimated ten times this number in reality from a ten percent reporting rate percentage. That's seven million adverse reactions and 10,000 potential deaths and a one percent reporting rate would be ten times *those* figures. In 1976 the US government pulled the swine flu vaccine after *53* deaths. The UK data included a combined 10,000 eye disorders from the 'Covid vaccines' with more than 750 suffering visual impairment or blindness and again multiply by the estimated reporting percentages. As 'Covid cases' officially fell hospitals virtually empty during the 'Covid crisis' began to fill up with a range of other problems in the wake of the 'vaccine' rollout. The numbers across America have also been catastrophic. Deaths linked to *all* types of vaccine increased by *6,000 percent* in the first quarter of 2021 compared with 2020. A 39-year-old woman from Ogden, Utah, died four days after receiving a second dose of Moderna's 'Covid vaccine' when her liver, heart and kidneys all failed despite the fact that she had no known medical issues or conditions. Her family sought an autopsy, but Dr Erik Christensen, Utah's chief medical examiner, said proving vaccine injury as a cause of death almost never happened. He could think of only one instance where an autopsy would name a vaccine as the official cause of death and that would be anaphylaxis where someone received a vaccine and died almost instantaneously. 'Short of that, it would be difficult for us to definitively say this is the vaccine,' Christensen said. If that is true this must be added to the estimated ten percent (or far less) reporting rate of vaccine deaths and serious reactions and the conclusion can only be that vaccine deaths and serious reactions – including these 'Covid' potions' – are phenomenally understated in official figures. The same story can be found everywhere. Endless accounts of deaths and serious reactions among the public, medical

and care home staff while official figures did not even begin to reflect this.

Professional script-reader Dr David Williams, a 'top public-health official' in Ontario, Canada, insulted our intelligence by claiming only four serious adverse reactions and no deaths from the more than 380,000 vaccine doses then given. This bore no resemblance to what people knew had happened in their owns circles and we had Dirk Huyer in charge of getting millions vaccinated in Ontario while at the same time he was Chief Coroner for the province investigating causes of death including possible death from the vaccine. An aide said he had stepped back from investigating deaths, but evidence indicated otherwise. Rosemary Frei, who secured a Master of Science degree in molecular biology at the Faculty of Medicine at Canada's University of Calgary before turning to investigative journalism, was one who could see that official figures for 'vaccine' deaths and reactions made no sense. She said that doctors seldom reported adverse events and when people got really sick or died after getting a vaccination they would attribute that to anything except the vaccines. It had been that way for years and anyone who wondered aloud whether the 'Covid vaccines' or other shots cause harm is immediately branded as 'anti-vax' and 'anti-science'. This was 'career-threatening' for health professionals. Then there was the huge pressure to support the push to 'vaccinate' billions in the quickest time possible. Frei said:

> So that's where we're at today. More than half a million vaccine doses have been given to people in Ontario alone. The rush is on to vaccinate all 15 million of us in the province by September. And the mainstream media are screaming for this to be sped up even more. That all adds up to only a very slim likelihood that we're going to be told the truth by officials about how many people are getting sick or dying from the vaccines.

What is true of Ontario is true of everywhere.

They KNEW – and still did it

The authorities knew what was going to happen with multiple deaths and adverse reactions. The UK government's Gates-funded

and Big Pharma-dominated Medicines and Healthcare products Regulatory Agency (MHRA) hired a company to employ AI in compiling the projected reactions to the 'vaccine' that would otherwise be uncountable. The request for applications said: 'The MHRA urgently seeks an Artificial Intelligence (AI) software tool to process the expected high volume of Covid-19 vaccine Adverse Drug Reaction …' This was from the agency, headed by the disingenuous June Raine, that gave the 'vaccines' emergency approval and the company was hired before the first shot was given. 'We are going to kill and maim you – is that okay?' 'Oh, yes, perfectly fine – I'm very grateful, thank you, doctor.' The range of 'Covid vaccine' adverse reactions goes on for page after page in the MHRA criminally underreported 'Yellow Card' system and includes affects to eyes, ears, skin, digestion, blood and so on. Raine's MHRA amazingly claimed that the 'overall safety experience … is so far as expected from the clinical trials'. The death, serious adverse effects, deafness and blindness were *expected?* When did they ever mention that? If these human tragedies were expected then those that gave approval for the use of these 'vaccines' must be guilty of crimes against humanity including murder – a definition of which is 'killing a person with malice aforethought or with recklessness manifesting extreme indifference to the value of human life.' People involved at the MHRA, the CDC in America and their equivalent around the world must go before Nuremberg trials to answer for their callous inhumanity. We are only talking here about the immediate effects of the 'vaccine'. The longer-term impact of the DNA synthetic manipulation is the main reason they are so hysterically desperate to inoculate the entire global population in the shortest possible time.

Africa and the developing world are a major focus for the 'vaccine' depopulation agenda and a mass vaccination sales-pitch is underway thanks to caring people like the Rockefellers and other Cult assets. The Rockefeller Foundation, which pre-empted the 'Covid pandemic' in a document published in 2010 that 'predicted' what happened a decade later, announced an initial $34.95 million grant in February, 2021, 'to ensure more equitable access to Covid-19

testing and vaccines' among other things in Africa in collaboration with '24 organizations, businesses, and government agencies'. The pan-Africa initiative would focus on 10 countries: Burkina Faso, Ethiopia, Ghana, Kenya, Nigeria, Rwanda, South Africa, Tanzania, Uganda, and Zambia'. Rajiv Shah, President of the Rockefeller Foundation and former administrator of CIA-controlled USAID, said that if Africa was not mass-vaccinated (to change the DNA of its people) it was a 'threat to all of humanity' and not fair on Africans. When someone from the Rockefeller Foundation says they want to do something to help poor and deprived people and countries it is time for a belly-laugh. They are doing this out of the goodness of their 'heart' because 'vaccinating' the entire global population is what the 'Covid' hoax set out to achieve. Official 'decolonisation' of Africa by the Cult was merely a prelude to financial colonisation on the road to a return to physical colonisation. The 'vaccine' is vital to that and the sudden and convenient death of the 'Covid' sceptic president of Tanzania can be seen in its true light. A lot of people in Africa are aware that this is another form of colonisation and exploitation and they need to stand their ground.

The 'vaccine is working' scam

A potential problem for the Cult was that the 'vaccine' is meant to change human DNA and body messaging and not to protect anyone from a 'virus' never shown to exist. The vaccine couldn't work because it was not designed to work and how could they make it *appear* to be working so that more people would have it? This was overcome by lowering the amplification rate of the PCR test to produce fewer 'cases' and therefore fewer 'deaths'. Some of us had been pointing out since March, 2020, that the amplification rate of the test not testing for the 'virus' had been made artificially high to generate positive tests which they could call 'cases' to justify lockdowns. The World Health Organization recommended an absurdly high 45 amplification cycles to ensure the high positives required by the Cult and then remained silent on the issue until January 20th, 2021 – Biden's Inauguration Day. This was when the

'vaccinations' were seriously underway and on that day the WHO recommended after discussions with America's CDC that laboratories *lowered their testing amplification*. Dr David Samadi, a certified urologist and health writer, said the WHO was encouraging all labs to reduce their cycle count for PCR tests. He said the current cycle was much too high and was 'resulting in any particle being declared a positive case'. Even one mainstream news report I saw said this meant the number of 'Covid' infections may have been 'dramatically inflated'. Oh, just a little bit. The CDC in America issued new guidance to laboratories in April, 2021, to use 28 cycles *but only for 'vaccinated' people*. The timing of the CDC/WHO interventions were cynically designed to make it appear the 'vaccines' were responsible for falling cases and deaths when the real reason can be seen in the following examples. New York's state lab, the Wadsworth Center, identified 872 positive tests in July, 2020, based on a threshold of 40 cycles. When the figure was lowered to 35 cycles *43 percent* of the 872 were no longer 'positives'. At 30 cycles the figure was 63 percent. A Massachusetts lab found that between *85 to 90 percent* of people who tested positive in July with a cycle threshold of 40 would be negative at 30 cycles, Ashish Jha, MD, director of the Harvard Global Health Institute, said: 'I'm really shocked that it could be that high … Boy, does it really change the way we need to be thinking about testing.' I'm shocked that I could see the obvious in the spring of 2020, with no medical background, and most medical professionals still haven't worked it out. No, that's not shocking – it's terrifying.

Three weeks after the WHO directive to lower PCR cycles the London *Daily Mail* ran this headline: 'Why ARE Covid cases plummeting? New infections have fallen 45% in the US and 30% globally in the past 3 weeks but experts say vaccine is NOT the main driver because only 8% of Americans and 13% of people worldwide have received their first dose.' They acknowledged that the drop could not be attributed to the 'vaccine', but soon this morphed throughout the media into the 'vaccine' has caused cases and deaths to fall when it was the PCR threshold. In December, 2020, there was

chaos at English Channel ports with truck drivers needing negative 'Covid' tests before they could board a ferry home for Christmas. The government wanted to remove the backlog as fast as possible and they brought in troops to do the 'testing'. Out of 1,600 drivers just *36* tested positive and the rest were given the all clear to cross the Channel. I guess the authorities thought that 36 was the least they could get away with without the unquestioning catching on. The amplification trick which most people believed in the absence of information in the mainstream applied more pressure on those refusing the 'vaccine' to succumb when it 'obviously worked'. The truth was the exact opposite with deaths in care homes soaring with the 'vaccine' and in Israel the term used was 'skyrocket'. A re-analysis of published data from the Israeli Health Ministry led by Dr Hervé Seligmann at the Medicine Emerging Infectious and Tropical Diseases at Aix-Marseille University found that Pfizer's 'Covid vaccine' killed 'about 40 times more [elderly] people than the disease itself would have killed' during a five-week vaccination period and *260 times* more younger people than would have died from the 'virus' even according to the manipulated 'virus' figures. Dr Seligmann and his co-study author, Haim Yativ, declared after reviewing the Israeli 'vaccine' death data: 'This is a new Holocaust.'

Then, in mid-April, 2021, after vast numbers of people worldwide had been 'vaccinated', the story changed with clear coordination. The UK government began to prepare the ground for more future lockdowns when Nuremberg-destined Boris Johnson told yet another whopper. He said that cases had fallen because of *lockdowns* not 'vaccines'. Lockdowns are irrelevant when *there is no 'virus'* and the test and fraudulent death certificates are deciding the number of 'cases' and 'deaths'. Study after study has shown that lockdowns don't work and instead kill and psychologically destroy people. Meanwhile in the United States Anthony Fauci and Rochelle Walensky, the ultra-Zionist head of the CDC, peddled the same line. More lockdown was the answer and not the 'vaccine', a line repeated on cue by the moron that is Canadian Prime Minister Justin Trudeau. Why all the hysteria to get everyone 'vaccinated' if lockdowns and

not 'vaccines' made the difference? None of it makes sense on the face of it. Oh, but it does. The Cult wants lockdowns *and* the 'vaccine' and if the 'vaccine' is allowed to be seen as the total answer lockdowns would no longer be justified when there are still livelihoods to destroy. 'Variants' and renewed upward manipulation of PCR amplification are planned to instigate never-ending lockdown *and* more 'vaccines'.

You *must* have it – we're desperate

Israel, where the Jewish and Arab population are ruled by the Sabbatian Cult, was the front-runner in imposing the DNA-manipulating 'vaccine' on its people to such an extent that Jewish refusers began to liken what was happening to the early years of Nazi Germany. This would seem to be a fantastic claim. Why would a government of Jewish people be acting like the Nazis did? If you realise that the Sabbatian Cult was behind the Nazis and that Sabbatians hate Jews the pieces start to fit and the question of why a 'Jewish' government would treat Jews with such callous disregard for their lives and freedom finds an answer. Those controlling the government of Israel *aren't Jewish* – they're Sabbatian. Israeli lawyer Tamir Turgal was one who made the Nazi comparison in comments to German lawyer Reiner Fuellmich who is leading a class action lawsuit against the psychopaths for crimes against humanity. Turgal described how the Israeli government was vaccinating children and pregnant women on the basis that there was no evidence that this was dangerous when they had no evidence that it *wasn't* dangerous either. They just had no evidence. This was medical experimentation and Turgal said this breached the Nuremberg Code about medical experimentation and procedures requiring informed consent and choice. Think about that. A Nuremberg Code developed because of Nazi experimentation on Jews and others in concentration camps by people like the evil-beyond-belief Josef Mengele is being breached by the *Israeli* government; but when you know that it's a *Sabbatian* government along with its intelligence and military agencies like Mossad, Shin Bet and the Israeli Defense Forces, and that Sabbatians

were the force behind the Nazis, the kaleidoscope comes into focus. What have we come to when Israeli Jews are suing their government for violating the Nuremberg Code by essentially making Israelis subject to a medical experiment using the controversial 'vaccines'? It's a shocker that this has to be done in the light of what happened in Nazi Germany. The Anshe Ha-Emet, or 'People of the Truth', made up of Israeli doctors, lawyers, campaigners and public, have launched a lawsuit with the International Criminal Court. It says:

> When the heads of the Ministry of Health as well as the prime minister presented the vaccine in Israel and began the vaccination of Israeli residents, the vaccinated were not advised, that, in practice, they are taking part in a medical experiment and that their consent is required for this under the Nuremberg Code.

The irony is unbelievable, but easily explained in one word: Sabbatians. The foundation of Israeli 'Covid' apartheid is the 'green pass' or 'green passport' which allows Jews and Arabs who have had the DNA-manipulating 'vaccine' to go about their lives – to work, fly, travel in general, go to shopping malls, bars, restaurants, hotels, concerts, gyms, swimming pools, theatres and sports venues, while non-'vaccinated' are banned from all those places and activities. Israelis have likened the 'green pass' to the yellow stars that Jews in Nazi Germany were forced to wear – the same as the yellow stickers that a branch of UK supermarket chain Morrisons told exempt mask-wears they had to display when shopping. How very sensitive. The Israeli system is blatant South African-style apartheid on the basis of compliance or non-compliance to fascism rather than colour of the skin. How appropriate that the Sabbatian Israeli government was so close to the pre-Mandela apartheid regime in Pretoria. The Sabbatian-instigated 'vaccine passport' in Israel is planned for everywhere. Sabbatians struck a deal with Pfizer that allowed them to lead the way in the percentage of a national population infused with synthetic material and the result was catastrophic. Israeli freedom activist Shai Dannon told me how chairs were appearing on beaches that said 'vaccinated only'. Health Minister Yuli Edelstein said that anyone unwilling or unable to get

the jabs that 'confer immunity' will be 'left behind'. The man's a liar. Not even the makers claim the 'vaccines' confer immunity. When you see those figures of 'vaccine' deaths these psychopaths were saying that you must take the chance the 'vaccine' will kill you or maim you while knowing it will change your DNA or lockdown for you will be permanent. That's fascism. The Israeli parliament passed a law to allow personal information of the non-vaccinated to be shared with local and national authorities for three months. This was claimed by its supporters to be a way to 'encourage' people to be vaccinated. Hadas Ziv from Physicians for Human Rights described this as a 'draconian law which crushed medical ethics and the patient rights'. But that's the idea, the Sabbatians would reply.

Your papers, please

Sabbatian Israel was leading what has been planned all along to be a global 'vaccine pass' called a 'green passport' without which you would remain in permanent lockdown restriction and unable to do anything. This is how badly – *desperately* – the Cult is to get everyone 'vaccinated'. The term and colour 'green' was not by chance and related to the psychology of fusing the perception of the green climate hoax with the 'Covid' hoax and how the 'solution' to both is the same Great Reset. Lying politicians, health officials and psychologists denied there were any plans for mandatory vaccinations or restrictions based on vaccinations, but they knew that was exactly what was meant to happen with governments of all countries reaching agreements to enforce a global system. 'Free' Denmark and 'free' Sweden unveiled digital vaccine certification. Cyprus, Czech Republic, Estonia, Greece, Hungary, Iceland, Italy, Poland, Portugal, Slovakia, and Spain have all committed to a vaccine passport system and the rest including the whole of the EU would follow. The satanic UK government will certainly go this way despite mendacious denials and at the time of writing it is trying to manipulate the public into having the 'vaccine' so they could go abroad on a summer holiday. How would that work without something to prove you had the synthetic toxicity injected into you?

Documents show that the EU's European Commission was moving towards 'vaccine certificates' in 2018 and 2019 before the 'Covid' hoax began. They knew what was coming. Abracadabra – Ursula von der Leyen, the German President of the Commission, announced in March, 2021, an EU 'Digital Green Certificate' – green again – to track the public's 'Covid status'. The passport sting is worldwide and the Far East followed the same pattern with South Korea ruling that only those with 'vaccination' passports – again the *green* pass – would be able to 'return to their daily lives'.

Bill Gates has been preparing for this 'passport' with other Cult operatives for years and beyond the paper version is a Gates-funded 'digital tattoo' to identify who has been vaccinated and who hasn't. The 'tattoo' is reported to include a substance which is externally readable to confirm who has been vaccinated. This is a bio-luminous light-generating enzyme (think fireflies) called … *Luciferase*. Yes, named after the Cult 'god' Lucifer the 'light bringer' of whom more to come. Gates said he funded the readable tattoo to ensure children in the developing world were vaccinated and no one was missed out. He cares so much about poor kids as we know. This was just the cover story to develop a vaccine tagging system for everyone on the planet. Gates has been funding the ID2020 'alliance' to do just that in league with other lovely people at Microsoft, GAVI, the Rockefeller Foundation, Accenture and IDEO.org. He said in interviews in March, 2020, before any 'vaccine' publicly existed, that the world must have a globalised digital certificate to track the 'virus' and who had been vaccinated. Gates knew from the start that the mRNA vaccines were coming and when they would come and that the plan was to tag the 'vaccinated' to marginalise the intelligent and stop them doing anything including travel. Evil just doesn't suffice. Gates was exposed for offering a $10 million bribe to the Nigerian House of Representatives to invoke compulsory 'Covid' vaccination of all Nigerians. Sara Cunial, a member of the Italian Parliament, called Gates a 'vaccine criminal'. She urged the Italian President to hand him over to the International Criminal Court for crimes against

humanity and condemned his plans to 'chip the human race' through ID2020.

You know it's a long-planned agenda when war criminal and Cult gofer Tony Blair is on the case. With the scale of arrogance only someone as dark as Blair can muster he said: 'Vaccination in the end is going to be your route to liberty.' Blair is a disgusting piece of work and he confirms that again. The media has given a lot of coverage to a bloke called Charlie Mullins, founder of London's biggest independent plumbing company, Pimlico Plumbers, who has said he won't employ anyone who has not been vaccinated or have them go to any home where people are not vaccinated. He said that if he had his way no one would be allowed to walk the streets if they have not been vaccinated. Gates was cheering at the time while I was alerting the white coats. The plan is that people will qualify for 'passports' for having the first two doses and then to keep it they will have to have all the follow ups and new ones for invented 'variants' until human genetics is transformed and many are dead who can't adjust to the changes. Hollywood celebrities – the usual propaganda stunt – are promoting something called the WELL Health-Safety Rating to verify that a building or space has 'taken the necessary steps to prioritize the health and safety of their staff, visitors and other stakeholders'. They included Lady Gaga, Jennifer Lopez, Michael B. Jordan, Robert DeNiro, Venus Williams, Wolfgang Puck, Deepak Chopra and 17th Surgeon General Richard Carmona. Yawn. WELL Health-Safety has big connections with China. Parent company Delos is headed by former Goldman Sachs partner Paul Scialla. This is another example – and we will see so many others – of using the excuse of 'health' to dictate the lives and activities of the population. I guess one confirmation of the 'safety' of buildings is that only 'vaccinated' people can go in, right?

Electronic concentration camps

I wrote decades ago about the plans to restrict travel and here we are for those who refuse to bow to tyranny. This can be achieved in one go with air travel if the aviation industry makes a blanket decree.

The ‘vaccine’ and guaranteed income are designed to be part of a global version of China’s social credit system which tracks behaviour 24/7 and awards or deletes ‘credits’ based on whether your behaviour is supported by the state or not. I mean your entire lifestyle – what you do, eat, say, everything. Once your credit score falls below a certain level consequences kick in. In China tens of millions have been denied travel by air and train because of this. All the locations and activities denied to refusers by the ‘vaccine’ passports will be included in one big mass ban on doing almost anything for those that don’t bow their head to government. It’s beyond fascist and a new term is required to describe its extremes – I guess fascist technocracy will have to do. The way the Chinese system of technological – technocratic – control is sweeping the West can be seen in the Los Angeles school system and is planned to be expanded worldwide. Every child is required to have a ‘Covid’-tracking app scanned daily before they can enter the classroom. The so-called Daily Pass tracking system is produced by Gates’ Microsoft which I’m sure will shock you rigid. The pass will be scanned using a barcode (one step from an inside-the-body barcode) and the information will include health checks, ‘Covid’ tests and vaccinations. Entry codes are for one specific building only and access will only be allowed if a student or teacher has a negative test with a test not testing for the ‘virus’, has no symptoms of anything alleged to be related to ‘Covid’ (symptoms from a range of other illness), and has a temperature under 100 degrees. No barcode, no entry, is planned to be the case for everywhere and not only schools.

Kids are being psychologically prepared to accept this as ‘normal’ their whole life which is why what they can impose in schools is so important to the Cult and its gofers. Long-time American freedom campaigner John Whitehead of the Rutherford Institute was not exaggerating when he said: ‘Databit by databit, we are building our own electronic concentration camps.’ Canada under its Cult gofer prime minister Justin Trudeau has taken a major step towards the real thing with people interned against their will if they test positive with a test not testing for the ‘virus’ when they arrive at a Canadian

airport. They are jailed in internment hotels often without food or water for long periods and with many doors failing to lock there have been sexual assaults. The interned are being charged sometimes $2,000 for the privilege of being abused in this way. Trudeau is fully on board with the Cult and says the 'Covid pandemic' has provided an opportunity for a global 'reset' to permanently change Western civilisation. His number two, Deputy Prime Minister Chrystia Freeland, is a trustee of the World Economic Forum and a Rhodes Scholar. The Trudeau family have long been servants of the Cult. See *The Biggest Secret* and Cathy O'Brien's book *Trance-Formation of America* for the horrific background to Trudeau's father Pierre Trudeau another Canadian prime minister. Hide your fascism behind the façade of a heart-on-the-sleeve liberal. It's a well-honed Cult technique.

What can the 'vaccine' *really* do?

We have a 'virus' never shown to exist and 'variants' of the 'virus' that have also never been shown to exist except, like the 'original', as computer-generated fictions. Even if you believe there's a 'virus' the 'case' to 'death' rate is in the region of 0.23 to 0.15 percent and those 'deaths' are concentrated among the very old around the same average age that people die anyway. In response to this lack of threat (in truth none) psychopaths and idiots, knowingly and unknowingly answering to Gates and the Cult, are seeking to 'vaccinate' every man, woman and child on Planet Earth. Clearly the 'vaccine' is not about 'Covid' – none of this ever has been. So what is it all about *really*? Why the desperation to infuse genetically-manipulating synthetic material into everyone through mRNA fraudulent 'vaccines' with the intent of doing this over and over with the excuses of 'variants' and other 'virus' inventions? Dr Sherri Tenpenny, an osteopathic medical doctor in the United States, has made herself an expert on vaccines and their effects as a vehement campaigner against their use. Tenpenny was board certified in emergency medicine, the director of a level two trauma centre for 12 years, and moved to Cleveland in 1996 to start an integrative

medicine practice which has treated patients from all 50 states and some 17 other countries. Weaning people off pharmaceutical drugs is a speciality.

She became interested in the consequences of vaccines after attending a meeting at the National Vaccine Information Center in Washington DC in 2000 where she 'sat through four days of listening to medical doctors and scientists and lawyers and parents of vaccine injured kids' and asked: 'What's going on?' She had never been vaccinated and never got ill while her father was given a list of vaccines to be in the military and was 'sick his entire life'. The experience added to her questions and she began to examine vaccine documents from the Centers for Disease Control (CDC). After reading the first one, the 1998 version of *The General Recommendations of Vaccination*, she thought: 'This is it?' The document was poorly written and bad science and Tenpenny began 20 years of research into vaccines that continues to this day. She began her research into 'Covid vaccines' in March, 2020, and she describes them as 'deadly'. For many, as we have seen, they already have been. Tenpenny said that in the first 30 days of the 'vaccine' rollout in the United States there had been more than 40,000 adverse events reported to the vaccine adverse event database. A document had been delivered to her the day before that was 172 pages long. 'We have over 40,000 adverse events; we have over 3,100 cases of [potentially deadly] anaphylactic shock; we have over 5,000 neurological reactions.' Effects ranged from headaches to numbness, dizziness and vertigo, to losing feeling in hands or feet and paraesthesia which is when limbs 'fall asleep' and people have the sensation of insects crawling underneath their skin. All this happened in the first 30 days and remember that only about *ten percent* (or far less) of adverse reactions and vaccine-related deaths are estimated to be officially reported. Tenpenny said:

> So can you think of one single product in any industry, any industry, for as long as products have been made on the planet that within 30 days we have 40,000 people complaining of side effects that not only is still on the market but … we've got paid actors telling us how great

they are for getting their vaccine. We're offering people $500 if they will just get their vaccine and we've got nurses and doctors going; 'I got the vaccine, I got the vaccine'.

Tenpenny said they were not going to be 'happy dancing folks' when they began to suffer Bell's palsy (facial paralysis), neuropathies, cardiac arrhythmias and autoimmune reactions that kill through a blood disorder. 'They're not going to be so happy, happy then, but we're never going to see pictures of those people' she said. Tenpenny described the 'vaccine' as 'a well-designed killing tool'.

No off-switch

Bad as the initial consequences had been Tenpenny said it would be maybe 14 months before we began to see the 'full ravage' of what is going to happen to the 'Covid vaccinated' with full-out consequences taking anything between two years and 20 years to show. You can understand why when you consider that variations of the 'Covid vaccine' use mRNA (messenger RNA) to in theory activate the immune system to produce protective antibodies without using the actual 'virus'. How can they when it's a computer program and they've never isolated what they claim is the 'real thing'? Instead they use *synthetic* mRNA. They are inoculating synthetic material into the body which through a technique known as the Trojan horse is absorbed into cells to change the nature of DNA. Human DNA is changed by an infusion of messenger RNA and with each new 'vaccine' of this type it is changed even more. Say so and you are banned by Cult Internet platforms. The contempt the contemptuous Mark Zuckerberg has for the truth and human health can be seen in an internal Facebook video leaked to the Project Veritas investigative team in which he said of the 'Covid vaccines': '… I share some caution on this because we just don't know the long term side-effects of basically modifying people's DNA and RNA.' At the same time this disgusting man's Facebook was censoring and banning anyone saying exactly the same. He must go before a Nuremberg trial for crimes against humanity when he *knows* that he

is censoring legitimate concerns and denying the right of informed consent on behalf of the Cult that owns him. People have been killed and damaged by the very 'vaccination' technique he cast doubt on himself when they may not have had the 'vaccine' with access to information that he denied them. The plan is to have at least annual 'Covid vaccinations', add others to deal with invented 'variants', and change all other vaccines into the mRNA system. Pfizer executives told shareholders at a virtual Barclays Global Healthcare Conference in March, 2021, that the public may need a third dose of 'Covid vaccine', plus regular yearly boosters and the company planned to hike prices to milk the profits in a 'significant opportunity for our vaccine'. These are the professional liars, cheats and opportunists who are telling you their 'vaccine' is safe. Given this volume of mRNA planned to be infused into the human body and its ability to then replicate we will have a transformation of human genetics from biological to synthetic biological – exactly the long-time Cult plan for reasons we'll see – and many will die. Sherri Tenpenny said of this replication:

> It's like having an on-button but no off-button and that whole mechanism … they actually give it a name and they call it the Trojan horse mechanism, because it allows that [synthetic] virus and that piece of that [synthetic] virus to get inside of your cells, start to replicate and even get inserted into other parts of your DNA as a Trojan-horse.

Ask the overwhelming majority of people who have the 'vaccine' what they know about the contents and what they do and they would reply: 'The government says it will stop me getting the virus.' Governments give that false impression on purpose to increase take-up. You can read Sherri Tenpenny's detailed analysis of the health consequences in her blog at Vaxxter.com, but in summary these are some of them. She highlights the statement by Bill Gates about how human beings can become their own 'vaccine manufacturing machine'. The man is insane. ['Vaccine'-generated] 'antibodies' carry synthetic messenger RNA into the cells and the damage starts, Tenpenny contends, and she says that lungs can be adversely affected through varying degrees of pus and bleeding which

obviously affects breathing and would be dubbed 'Covid-19'. Even more sinister was the impact of 'antibodies' on macrophages, a white blood cell of the immune system. They consist of Type 1 and Type 2 which have very different functions. She said Type 1 are 'hyper-vigilant' white blood cells which 'gobble up' bacteria etc. However, in doing so, this could cause inflammation and in extreme circumstances be fatal. She says these affects are mitigated by Type 2 macrophages which kick in to calm down the system and stop it going rogue. They clear up dead tissue debris and reduce inflammation that the Type 1 'fire crews' have caused. Type 1 kills the infection and Type 2 heals the damage, she says. This is her punchline with regard to 'Covid vaccinations': She says that mRNA 'antibodies' block Type 2 macrophages by attaching to them and deactivating them. This meant that when the Type 1 response was triggered by infection there was nothing to stop that getting out of hand by calming everything down. There's an on-switch, but no off-switch, she says. What follows can be 'over and out, see you when I see you'.

Genetic suicide

Tenpenny also highlights the potential for autoimmune disease – the body attacking itself – which has been associated with vaccines since they first appeared. Infusing a synthetic foreign substance into cells could cause the immune system to react in a panic believing that the body is being overwhelmed by an invader (it is) and the consequences can again be fatal. There is an autoimmune response known as a 'cytokine storm' which I have likened to a homeowner panicked by an intruder and picking up a gun to shoot randomly in all directions before turning the fire on himself. The immune system unleashes a storm of inflammatory response called cytokines to a threat and the body commits hara-kiri. The lesson is that you mess with the body's immune response at your peril and these 'vaccines' seriously – fundamentally – mess with immune response. Tenpenny refers to a consequence called anaphylactic shock which is a severe and highly dangerous allergic reaction when the immune system

floods the body with chemicals. She gives the example of having a bee sting which primes the immune system and makes it sensitive to those chemicals. When people are stung again maybe years later the immune response can be so powerful that it leads to anaphylactic shock. Tenpenny relates this 'shock' with regard to the 'Covid vaccine' to something called polyethylene glycol or PEG. Enormous numbers of people have become sensitive to this over decades of use in a whole range of products and processes including food, drink, skin creams and 'medicine'. Studies have claimed that some 72 percent of people have antibodies triggered by PEG compared with two percent in the 1960s and allergic hypersensitive reactions to this become a gathering cause for concern. Tenpenny points out that the 'mRNA vaccine' is coated in a 'bubble' of polyethylene glycol which has the potential to cause anaphylactic shock through immune sensitivity. Many reports have appeared of people reacting this way after having the 'Covid vaccine'. What do we think is going to happen as humanity has more and more of these 'vaccines'? Tenpenny said: 'All these pictures we have seen with people with these rashes … these weepy rashes, big reactions on their arms and things like that – it's an acute allergic reaction most likely to the polyethylene glycol that you've been previously primed and sensitised to.'

Those who have not studied the conspiracy and its perpetrators at length might think that making the population sensitive to PEG and then putting it in these 'vaccines' is just a coincidence. It is not. It is instead testament to how carefully and coldly-planned current events have been and the scale of the conspiracy we are dealing with. Tenpenny further explains that the 'vaccine' mRNA procedure can breach the blood-brain barrier which protects the brain from toxins and other crap that will cause malfunction. In this case they could make two proteins corrupt brain function to cause Amyotrophic lateral sclerosis (ALS) , a progressive nervous system disease leading to loss of muscle control, and frontal lobe degeneration – Alzheimer's and dementia. Immunologist J. Bart Classon published a paper connecting mRNA 'vaccines' to prion

disease which can lead to Alzheimer's and other forms of neurogenerative disease while others have pointed out the potential to affect the placenta in ways that make women infertile. This will become highly significant in the next chapter when I will discuss other aspects of this non-vaccine that relate to its nanotechnology and transmission from the injected to the uninjected.

Qualified in idiocy

Tenpenny describes how research has confirmed that these 'vaccine'-generated antibodies can interact with a range of other tissues in the body and attack many other organs including the lungs. 'This means that if you have a hundred people standing in front of you that all got this shot they could have a hundred different symptoms.' Anyone really think that Cult gofers like the Queen, Tony Blair, Christopher Whitty, Anthony Fauci, and all the other psychopaths have really had this 'vaccine' in the pictures we've seen? Not a bloody chance. Why don't doctors all tell us about all these dangers and consequences of the 'Covid vaccine'? Why instead do they encourage and pressure patients to have the shot? Don't let's think for a moment that doctors and medical staff can't be stupid, lazy, and psychopathic and that's without the financial incentives to give the jab. Tenpenny again:

> Some people are going to die from the vaccine directly but a large number of people are going to start to get horribly sick and get all kinds of autoimmune diseases 42 days to maybe a year out. What are they going to do, these stupid doctors who say; 'Good for you for getting that vaccine.' What are they going to say; 'Oh, it must be a mutant, we need to give an extra dose of that vaccine.'
>
> Because now the vaccine, instead of one dose or two doses we need three or four because the stupid physicians aren't taking the time to learn anything about it. If I can learn this sitting in my living room reading a 19 page paper and several others so can they. There's nothing special about me, I just take the time to do it.

Remember how Sara Kayat, the NHS and TV doctor, said that the 'Covid vaccine' would '100 percent prevent hospitalisation and death'. Doctors can be idiots like every other profession and they

should not be worshipped as infallible. They are not and far from it. Behind many medical and scientific 'experts' lies an uninformed prat trying to hide themselves from you although in the 'Covid' era many have failed to do so as with UK narrative-repeating 'TV doctor' Hilary Jones. Pushing back against the minority of proper doctors and scientists speaking out against the 'vaccine' has been the entire edifice of the Cult global state in the form of governments, medical systems, corporations, mainstream media, Silicon Valley, and an army of compliant doctors, medical staff and scientists willing to say anything for money and to enhance their careers by promoting the party line. If you do that you are an 'expert' and if you won't you are an 'anti-vaxxer' and 'Covidiot'. The pressure to be 'vaccinated' is incessant. We have even had reports claiming that the 'vaccine' can help cure cancer and Alzheimer's and make the lame walk. I am waiting for the announcement that it can bring you coffee in the morning and cook your tea. Just as the symptoms of 'Covid' seem to increase by the week so have the miracles of the 'vaccine'. American supermarket giant Kroger Co. offered nearly 500,000 employees in 35 states a $100 bonus for having the 'vaccine' while donut chain Krispy Kreme promised 'vaccinated' customers a free glazed donut every day for the rest of 2021. Have your DNA changed and you will get a doughnut although we might not have to give you them for long. Such offers and incentives confirm the desperation.

Perhaps the worse vaccine-stunt of them all was UK 'Health' Secretary Matt-the-prat Hancock on live TV after watching a clip of someone being 'vaccinated' when the roll-out began. Hancock faked tears so badly it was embarrassing. Brain-of-Britain Piers Morgan, the lockdown-supporting, 'vaccine' supporting, 'vaccine' passport-supporting, TV host played along with Hancock – 'You're quite emotional about that' he said in response to acting so atrocious it would have been called out at a school nativity which will presumably today include Mary and Jesus in masks, wise men keeping their camels six feet apart, and shepherds under tent arrest. System-serving Morgan tweeted this: 'Love the idea of covid vaccine passports for everywhere: flights, restaurants, clubs, football, gyms,

shops etc. It's time covid-denying, anti-vaxxer loonies had their bullsh*t bluff called & bar themselves from going anywhere that responsible citizens go.' If only I could aspire to his genius. To think that Morgan, who specialises in shouting over anyone he disagrees with, was lauded as a free speech hero when he lost his job after storming off the set of his live show like a child throwing his dolly out of the pram. If he is a free speech hero we are in real trouble. I have no idea what 'bullsh*t' means, by the way, the * throws me completely.

The Cult is desperate to infuse its synthetic DNA-changing concoction into everyone and has been using every lie, trick and intimidation to do so. The question of '*Why*?' we shall now address.

CHAPTER TEN

Human 2.0

I believe that at the end of the century the use of words and general educated opinion will have altered so much that one will be able to speak of machines thinking without expecting to be contradicted –
Alan Turing (1912-1954), the 'Father of artificial intelligence'

I have been exposing for decades the plan to transform the human body from a biological to a synthetic-biological state. The new human that I will call Human 2.0 is planned to be connected to artificial intelligence and a global AI 'Smart Grid' that would operate as one global system in which AI would control everything from your fridge to your heating system to your car to your mind. Humans would no longer be 'human', but post-human and sub-human, with their thinking and emotional processes replaced by AI.

What I said sounded crazy and beyond science fiction and I could understand that. To any balanced, rational, mind it *is* crazy. Today, however, that world is becoming reality and it puts the 'Covid vaccine' into its true context. Ray Kurzweil is the ultra-Zionist 'computer scientist, inventor and futurist' and co-founder of the Singularity University. Singularity refers to the merging of humans with machines or 'transhumanism'. Kurzweil has said humanity would be connected to the cyber 'cloud' in the period of the ever-recurring year of 2030:

Our thinking … will be a hybrid of biological and non-biological thinking … humans will be able to extend their limitations and 'think in the cloud' … We're going to put gateways to the

cloud in our brains ... We're going to gradually merge and enhance ourselves ... In my view, that's the nature of being human – we transcend our limitations. As the technology becomes vastly superior to what we are then the small proportion that is still human gets smaller and smaller and smaller until it's just utterly negligible.

They are trying to sell this end-of-humanity-as-we-know-it as the next stage of 'evolution' when we become super-human and 'like the gods'. They are lying to you. Shocked, eh? The population, and again especially the young, have been manipulated into addiction to technologies designed to enslave them for life. First they induced an addiction to smartphones (holdables); next they moved to technology on the body (wearables); and then began the invasion of the body (implantables). I warned way back about the plan for microchipped people and we are now entering that era. We should not be diverted into thinking that this refers only to chips we can see. Most important are the nanochips known as smart dust, neural dust and nanobots which are far too small to be seen by the human eye. Nanotechnology is everywhere, increasingly in food products, and released into the atmosphere by the geoengineering of the skies funded by Bill Gates to 'shut out the Sun' and 'save the planet from global warming'. Gates has been funding a project to spray millions of tonnes of chalk (calcium carbonate) into the stratosphere over Sweden to 'dim the Sun' and cool the Earth. Scientists warned the move could be disastrous for weather systems in ways no one can predict and opposition led to the Swedish space agency announcing that the 'experiment' would not be happening as planned in the summer of 2021; but it shows where the Cult is going with dimming the impact of the Sun and there's an associated plan to change the planet's atmosphere. Who gives psychopath Gates the right to dictate to the entire human race and dismantle planetary systems? The world will not be safe while this man is at large.

The global warming hoax has made the Sun, like the gas of life, something to fear when both are essential to good health and human survival (more inversion). The body transforms sunlight into vital vitamin D through a process involving … *cholesterol*. This is the cholesterol we are also told to fear. We are urged to take Big Pharma

statin drugs to reduce cholesterol and it's all systematic. Reducing cholesterol means reducing vitamin D uptake with all the multiple health problems that will cause. At least if you take statins long term it saves the government from having to pay you a pension. The delivery system to block sunlight is widely referred to as chemtrails although these have a much deeper agenda, too. They appear at first to be contrails or condensation trails streaming from aircraft into cold air at high altitudes. Contrails disperse very quickly while chemtrails do not and spread out across the sky before eventually their content falls to earth. Many times I have watched aircraft cross-cross a clear blue sky releasing chemtrails until it looks like a cloudy day. Chemtrails contain many things harmful to humans and the natural world including toxic heavy metals, aluminium (see Alzheimer's) and nanotechnology. Ray Kurzweil reveals the reason without actually saying so: 'Nanobots will infuse all the matter around us with information. Rocks, trees, everything will become these intelligent creatures.' How do you deliver that? *From the sky*. Self-replicating nanobots would connect everything to the Smart Grid. The phenomenon of Morgellons disease began in the chemtrail era and the correlation has led to it being dubbed the 'chemtrail disease'. Self-replicating fibres appear in the body that can be pulled out through the skin. Morgellons fibres continue to grow outside the body and have a form of artificial intelligence. I cover this at greater length in *Phantom Self*.

'Vaccine' operating system

'Covid vaccines' with their self-replicating synthetic material are also designed to make the connection between humanity and Kurzweil's 'cloud'. American doctor and dedicated campaigner for truth, Carrie Madej, an Internal Medicine Specialist in Georgia with more than 20 years medical experience, has highlighted the nanotechnology aspect of the fake 'vaccines'. She explains how one of the components in at least the Moderna and Pfizer synthetic potions are 'lipid nanoparticles' which are 'like little tiny computer bits' – a 'sci-fi substance' known as nanobots and hydrogel which can be 'triggered

at any moment to deliver its payload' and act as 'biosensors'. The synthetic substance had 'the ability to accumulate data from your body like your breathing, your respiration, thoughts and emotions, all kind of things' and each syringe could carry a *million* nanobots:

This substance because it's like little bits of computers in your body, crazy, but it's true, it can do that, [and] obviously has the ability to act through Wi-Fi. It can receive and transmit energy, messages, frequencies or impulses. That issue has never been addressed by these companies. What does that do to the human?

Just imagine getting this substance in you and it can react to things all around you, the 5G, your smart device, your phones, what is happening with that? What if something is triggering it, too, like an impulse, a frequency? We have something completely foreign in the human body.

Madej said her research revealed that electromagnetic (EMF) frequencies emitted by phones and other devices had increased dramatically in the same period of the 'vaccine' rollout and she was seeing more people with radiation problems as 5G and other electromagnetic technology was expanded and introduced to schools and hospitals. She said she was 'floored with the EMF coming off' the devices she checked. All this makes total sense and syncs with my own work of decades when you think that Moderna refers in documents to its mRNA 'vaccine' as an 'operating system':

Recognizing the broad potential of mRNA science, we set out to create an mRNA technology platform that functions very much like an operating system on a computer. It is designed so that it can plug and play interchangeably with different programs. In our case, the 'program' or 'app' is our mRNA drug – the unique mRNA sequence that codes for a protein …

… Our MRNA Medicines – 'The 'Software Of Life': When we have a concept for a new mRNA medicine and begin research, fundamental components are already in place. Generally, the only thing that changes from one potential mRNA medicine to another is the coding region – the actual genetic code that instructs ribosomes to make protein. Utilizing these instruction sets gives our investigational mRNA medicines a software-like quality. We also have the ability to combine different mRNA sequences encoding for different proteins in a single mRNA investigational medicine.

Who needs a real 'virus' when you can create a computer version to justify infusing your operating system into the entire human race on the road to making living, breathing people into cyborgs? What is missed with the 'vaccines' is the *digital* connection between synthetic material and the body that I highlighted earlier with the study that hacked a computer with human DNA. On one level the body is digital, based on mathematical codes, and I'll have more about that in the next chapter. Those who ridiculously claim that mRNA 'vaccines' are not designed to change human genetics should explain the words of Dr Tal Zaks, chief medical officer at Moderna, in a 2017 TED talk. He said that over the last 30 years 'we've been living this phenomenal digital scientific revolution, and I'm here today to tell you, that we are actually *hacking the software of life*, and that it's changing the way we think about prevention and treatment of disease':

In every cell there's this thing called messenger RNA, or mRNA for short, that transmits the critical information from the DNA in our genes to the protein, which is really the stuff we're all made out of. This is the critical information that determines what the cell will do. So we think about it as an operating system. So if you could change that, if you could introduce a line of code, or change a line of code, it turns out, that has profound implications for everything, from the flu to cancer.

Zaks should more accurately have said that this has profound implications for the human genetic code and the nature of DNA. Communications within the body go both ways and not only one. But, hey, no, the 'Covid vaccine' will not affect your genetics. Cult fact-checkers say so even though the man who helped to develop the mRNA technique says that it does. Zaks said in 2017:

If you think about what it is we're trying to do. We've taken information and our understanding of that information and how that information is transmitted in a cell, and we've taken our understanding of medicine and how to make drugs, and we're fusing the two. We think of it as information therapy.

I have been writing for decades that the body is an information field communicating with itself and the wider world. This is why

radiation which is information can change the information field of body and mind through phenomena like 5G and change their nature and function. 'Information therapy' means to change the body's information field and change the way it operates. DNA is a receiver-transmitter of information and can be mutated by information like mRNA synthetic messaging. Technology to do this has been ready and waiting in the underground bases and other secret projects to be rolled out when the 'Covid' hoax was played. 'Trials' of such short and irrelevant duration were only for public consumption. When they say the 'vaccine' is 'experimental' that is not true. It may appear to be 'experimental' to those who don't know what's going on, but the trials have already been done to ensure the Cult gets the result it desires. Zaks said that it took decades to sequence the human genome, completed in 2003, but now they could do it in a week. By 'they' he means scientists operating in the public domain. In the secret projects they were sequencing the genome in a week long before even 2003.

Deluge of mRNA

Highly significantly the Moderna document says the guiding premise is that if using mRNA as a medicine works for one disease then it should work for many diseases. They were leveraging the flexibility afforded by their platform and the fundamental role mRNA plays in protein synthesis to pursue mRNA medicines for a broad spectrum of diseases. Moderna is confirming what I was saying through 2020 that multiple 'vaccines' were planned for 'Covid' (and later invented 'variants') and that previous vaccines would be converted to the mRNA system to infuse the body with massive amounts of genetically-manipulating synthetic material to secure a transformation to a synthetic-biological state. The 'vaccines' are designed to kill stunning numbers as part of the long-exposed Cult depopulation agenda and transform the rest. Given this is the goal you can appreciate why there is such hysterical demand for every human to be 'vaccinated' for an alleged 'disease' that has an estimated 'infection' to 'death' ratio of 0.23-0.15 percent. As I write

children are being given the 'vaccine' in trials (their parents are a disgrace) and ever-younger people are being offered the vaccine for a 'virus' that even if you believe it exists has virtually zero chance of harming them. Horrific effects of the 'trials' on a 12-year-old girl were revealed by a family member to be serious brain and gastric problems that included a bowel obstruction and the inability to swallow liquids or solids. She was unable to eat or drink without throwing up, had extreme pain in her back, neck and abdomen, and was paralysed from the waist down which stopped her urinating unaided. When the girl was first taken to hospital doctors said it was all in her mind. She was signed up for the 'trial' by her parents for whom no words suffice. None of this 'Covid vaccine' insanity makes any sense unless you see what the 'vaccine' really is – a body-changer. Synthetic biology or 'SynBio' is a fast-emerging and expanding scientific discipline which includes everything from genetic and molecular engineering to electrical and computer engineering. Synthetic biology is defined in these ways:

- A multidisciplinary area of research that seeks to create new biological parts, devices, and systems, or to redesign systems that are already found in nature.

- The use of a mixture of physical engineering and genetic engineering to create new (and therefore synthetic) life forms.

- An emerging field of research that aims to combine the knowledge and methods of biology, engineering and related disciplines in the design of chemically-synthesized DNA to create organisms with novel or enhanced characteristics and traits (synthetic organisms including humans).

We now have synthetic blood, skin, organs and limbs being developed along with synthetic body parts produced by 3D printers. These are all elements of the synthetic human programme and this comment by Kurzweil's co-founder of the Singularity University,

Peter Diamandis, can be seen in a whole new light with the 'Covid' hoax and the sanctions against those that refuse the 'vaccine':

Anybody who is going to be resisting the progress forward [to transhumanism] is going to be resisting evolution and, fundamentally, they will die out. It's not a matter of whether it's good or bad. It's going to happen.

'Resisting evolution'? What absolute bollocks. The arrogance of these people is without limit. His 'it's going to happen' mantra is another way of saying 'resistance is futile' to break the spirit of those pushing back and we must not fall for it. Getting this genetically-transforming 'vaccine' into everyone is crucial to the Cult plan for total control and the desperation to achieve that is clear for anyone to see. Vaccine passports are a major factor in this and they, too, are a form of resistance is futile. It's NOT. The paper funded by the Rockefeller Foundation for the 2013 'health conference' in China said:

We will interact more with artificial intelligence. The use of robotics, bio-engineering to augment human functioning is already well underway and will advance. Re-engineering of humans into potentially separate and unequal forms through genetic engineering or mixed human-robots raises debates on ethics and equality.

A new demography is projected to emerge after 2030 [that year again] of technologies (robotics, genetic engineering, nanotechnology) producing robots, engineered organisms, 'nanobots' and artificial intelligence (AI) that can self-replicate. Debates will grow on the implications of an impending reality of human designed life.

What is happening today is so long planned. The world army enforcing the will of the world government is intended to be a robot army, not a human one. Today's military and its technologically 'enhanced' troops, pilotless planes and driverless vehicles are just stepping stones to that end. Human soldiers are used as Cult fodder and its time they woke up to that and worked for the freedom of the population instead of their own destruction and their family's destruction – the same with the police. Join us and let's sort this out. The phenomenon of enforce my own destruction is widespread in the 'Covid' era with Woker 'luvvies' in the acting and entertainment

industries supporting 'Covid' rules which have destroyed their profession and the same with those among the public who put signs on the doors of their businesses 'closed due to Covid – stay safe' when many will never reopen. It's a form of masochism and most certainly insanity.

Transgender = transhumanism

When something explodes out of nowhere and is suddenly everywhere it is always the Cult agenda and so it is with the tidal wave of claims and demands that have infiltrated every aspect of society under the heading of 'transgenderism'. The term 'trans' is so 'in' and this is the dictionary definition:

A prefix meaning 'across', 'through', occurring … in loanwords from Latin, used in particular for denoting movement or conveyance from place to place (transfer; transmit; transplant) or complete change (transform; transmute), or to form adjectives meaning 'crossing', 'on the other side of', or 'going beyond' the place named (transmontane; transnational; trans-Siberian).

Transgender means to go beyond gender and transhuman means to go beyond human. Both are aspects of the Cult plan to transform the human body to a synthetic state with *no gender*. Human 2.0 is not designed to procreate and would be produced technologically with no need for parents. The new human would mean the end of parents and so men, and increasingly women, are being targeted for the deletion of their rights and status. Parental rights are disappearing at an ever-quickening speed for the same reason. The new human would have no need for men or women when there is no procreation and no gender. Perhaps the transgender movement that appears to be in a permanent state of frenzy might now contemplate on how it is being used. This was never about transgender rights which are only the interim excuse for confusing gender, particularly in the young, on the road to *fusing* gender. Transgender activism is not an end; it is a *means* to an end. We see again the technique of creative destruction in which you destroy the status quo to 'build back better' in the form that you want. The gender status quo had to be

destroyed by persuading the Cult-created Woke mentality to believe that you can have 100 genders or more. A programme for 9 to 12 year olds produced by the Cult-owned BBC promoted the 100 genders narrative. The very idea may be the most monumental nonsense, but it is not what is true that counts, only what you can make people *believe* is true. Once the gender of 2 + 2 = 4 has been dismantled through indoctrination, intimidation and 2 + 2 = 5 then the new no-gender normal can take its place with Human 2.0. Aldous Huxley revealed the plan in his prophetic *Brave New World* in 1932:

> Natural reproduction has been done away with and children are created, decanted', and raised in 'hatcheries and conditioning centres'. From birth, people are genetically designed to fit into one of five castes, which are further split into 'Plus' and 'Minus' members and designed to fulfil predetermined positions within the social and economic strata of the World State.

How could Huxley know this in 1932? For the same reason George Orwell knew about the Big Brother state in 1948, Cult insiders I have quoted knew about it in 1969, and I have known about it since the early 1990s. If you are connected to the Cult or you work your balls off to uncover the plan you can predict the future. The process is simple. If there is a plan for the world and nothing intervenes to stop it then it will happen. Thus if you communicate the plan ahead of time you are perceived to have predicted the future, but you haven't. You have revealed the plan which without intervention will become the human future. The whole reason I have done what I have is to alert enough people to inspire an intervention and maybe at last that time has come with the Cult and its intentions now so obvious to anyone with a brain in working order.

The future is here

Technological wombs that Huxley described to replace parent procreation are already being developed and they are only the projects we know about in the public arena. Israeli scientists told *The Times of Israel* in March, 2021, that they have grown 250-cell embryos

into mouse foetuses with fully formed organs using artificial wombs in a development they say could pave the way for gestating humans outside the womb. Professor Jacob Hanna of the Weizmann Institute of Science said:

We took mouse embryos from the mother at day five of development, when they are just of 250 cells, and had them in the incubator from day five until day 11, by which point they had grown all their organs.

By day 11 they make their own blood and have a beating heart, a fully developed brain. Anybody would look at them and say, 'this is clearly a mouse foetus with all the characteristics of a mouse.' It's gone from being a ball of cells to being an advanced foetus.

A special liquid is used to nourish embryo cells in a laboratory dish and they float on the liquid to duplicate the first stage of embryonic development. The incubator creates all the right conditions for its development, Hanna said. The liquid gives the embryo 'all the nutrients, hormones and sugars they need' along with a custom-made electronic incubator which controls gas concentration, pressure and temperature. The cutting-edge in the underground bases and other secret locations will be light years ahead of that, however, and this was reported by the London *Guardian* in 2017:

We are approaching a biotechnological breakthrough. Ectogenesis, the invention of a complete external womb, could completely change the nature of human reproduction. In April this year, researchers at the Children's Hospital of Philadelphia announced their development of an artificial womb.

The article was headed 'Artificial wombs could soon be a reality. What will this mean for women?' What would it mean for children is an even bigger question. No mother to bond with only a machine in preparation for a life of soulless interaction and control in a world governed by machines (see the *Matrix* movies). Now observe the calculated manipulations of the 'Covid' hoax as human interaction and warmth has been curtailed by distancing, isolation and fear with people communicating via machines on a scale never seen before.

These are all dots in the same picture as are all the personal assistants, gadgets and children's toys through which kids and adults communicate with AI as if it is human. The AI 'voice' on Sat-Nav should be included. All these things are psychological preparation for the Cult endgame. Before you can make a physical connection with AI you have to make a psychological connection and that is what people are being conditioned to do with this ever gathering human-AI interaction. Movies and TV programmes depicting the transhuman, robot dystopia relate to a phenomenon known as 'pre-emptive programming' in which the world that is planned is portrayed everywhere in movies, TV and advertising. This is conditioning the conscious and subconscious mind to become familiar with the planned reality to dilute resistance when it happens for real. What would have been a shock such is the change is made less so. We have young children put on the road to transgender transition surgery with puberty blocking drugs at an age when they could never be able to make those life-changing decisions.

Rachel Levine, a professor of paediatrics and psychiatry who believes in treating children this way, became America's highest-ranked openly-transgender official when she was confirmed as US Assistant Secretary at the Department of Health and Human Services after being nominated by Joe Biden (the Cult). Activists and governments press for laws to deny parents a say in their children's transition process so the kids can be isolated and manipulated into agreeing to irreversible medical procedures. A Canadian father Robert Hoogland was denied bail by the Vancouver Supreme Court in 2021 and remained in jail for breaching a court order that he stay silent over his young teenage daughter, a minor, who was being offered life-changing hormone therapy without parental consent. At the age of 12 the girl's 'school counsellor' said she may be transgender, referred her to a doctor and told the school to treat her like a boy. This is another example of state-serving schools imposing ever more control over children's lives while parents have ever less.

Contemptible and extreme child abuse is happening all over the world as the Cult gender-fusion operation goes into warp-speed.

Why the war on men – and now women?

The question about what artificial wombs mean for women should rightly be asked. The answer can be seen in the deletion of women's rights involving sport, changing rooms, toilets and status in favour of people in male bodies claiming to identify as women. I can identify as a mountain climber, but it doesn't mean I can climb a mountain any more than a biological man can be a biological woman. To believe so is a triumph of belief over factual reality which is the very perceptual basis of everything Woke. Women's sport is being destroyed by allowing those with male bodies who say they identify as female to 'compete' with girls and women. Male body 'women' dominate 'women's' competition with their greater muscle mass, bone density, strength and speed. With that disadvantage sport for women loses all meaning. To put this in perspective nearly 300 American high school boys can run faster than the quickest woman sprinter in the world. Women are seeing their previously protected spaces invaded by male bodies simply because they claim to identify as women. That's all they need to do to access all women's spaces and activities under the Biden 'Equality Act' that destroys equality for women with the usual Orwellian Woke inversion. Male sex offenders have already committed rapes in women's prisons after claiming to identify as women to get them transferred. Does this not matter to the Woke 'equality' hypocrites? Not in the least. What matters to Cult manipulators and funders behind transgender activists is to advance gender fusion on the way to the no-gender 'human'. When you are seeking to impose transparent nonsense like this, or the 'Covid' hoax, the only way the nonsense can prevail is through censorship and intimidation of dissenters, deletion of factual information, and programming of the unquestioning, bewildered and naive. You don't have to scan the world for long to see that all these things are happening.

Many women's rights organisations have realised that rights and status which took such a long time to secure are being eroded and that it is systematic. Kara Dansky of the global Women's Human Rights Campaign said that Biden's transgender executive order immediately he took office, subsequent orders, and Equality Act legislation that followed 'seek to erase women and girls in the law as a category'. *Exactly*. I said during the long ago-started war on men (in which many women play a crucial part) that this was going to turn into a war on them. The Cult is phasing out *both* male and female genders. To get away with that they are brought into conflict so they are busy fighting each other while the Cult completes the job with no unity of response. Unity, people, *unity*. We need unity everywhere. Transgender is the only show in town as the big step towards the no-gender human. It's not about rights for transgender people and never has been. Woke political correctness is deleting words relating to genders to the same end. Wokers believe this is to be 'inclusive' when the opposite is true. They are deleting words describing gender because gender *itself* is being deleted by Human 2.0. Terms like 'man', 'woman', 'mother' and 'father' are being deleted in the universities and other institutions to be replaced by the *no*-gender, not trans-gender, 'individuals' and 'guardians'. Women's rights campaigner Maria Keffler of Partners for Ethical Care said: 'Children are being taught from kindergarten upward that some boys have a vagina, some girls have a penis, and that kids can be any gender they want to be.' Do we really believe that suddenly countries all over the world at the same time had the idea of having drag queens go into schools or read transgender stories to very young children in the local library? It's coldly-calculated confusion of gender on the way to the fusion of gender. Suzanne Vierling, a psychologist from Southern California, made another important point:

Yesterday's slave woman who endured gynecological medical experiments is today's girl-child being butchered in a booming gender-transitioning sector. Ovaries removed, pushing her into menopause and osteoporosis, uncharted territory, and parents' rights and authority decimated.

The erosion of parental rights is a common theme in line with the Cult plans to erase the very concept of parents and 'ovaries removed, pushing her into menopause' means what? Those born female lose the ability to have children – another way to discontinue humanity as we know it.

Eliminating Human 1.0 (before our very eyes)

To pave the way for Human 2.0 you must phase out Human 1.0. This is happening through plummeting sperm counts and making women infertile through an onslaught of chemicals, radiation (including smartphones in pockets of men) and mRNA 'vaccines'. Common agriculture pesticides are also having a devastating impact on human fertility. I have been tracking collapsing sperm counts in the books for a long time and in 2021 came a book by fertility scientist and reproductive epidemiologist Shanna Swan, *Count Down: How Our Modern World Is Threatening Sperm Counts, Altering Male and Female Reproductive Development and Imperiling the Future of the Human Race*. She reports how the global fertility rate dropped by *half* between 1960 and 2016 with America's birth rate 16 percent below where it needs to be to sustain the population. Women are experiencing declining egg quality, more miscarriages, and more couples suffer from infertility. Other findings were an increase in erectile dysfunction, infant boys developing more genital abnormalities, male problems with conception, and plunging levels of the male hormone testosterone which would explain why so many men have lost their backbone and masculinity. This has been very evident during the 'Covid' hoax when women have been prominent among the Pushbackers and big strapping blokes have bowed their heads, covered their faces with a nappy and quietly submitted. Mind control expert Cathy O'Brien also points to how global education introduced the concept of 'we're all winners' in sport and classrooms: 'Competition was defused, and it in turn defused a sense of fighting back.' This is another version of the 'equity' doctrine in which you drive down rather than raise up. What a contrast in Cult-controlled China with its global ambitions

where the government published plans in January, 2021, to 'cultivate masculinity' in boys from kindergarten through to high school in the face of a 'masculinity crisis'. A government adviser said boys would be soon become 'delicate, timid and effeminate' unless action was taken. Don't expect any similar policy in the targeted West. A 2006 study showed that a 65-year-old man in 2002 had testosterone levels *15 percent* lower than a 65-year-old man in 1987 while a 2020 study found a similar story with young adults and adolescents. Men are getting prescriptions for testosterone replacement therapy which causes an even greater drop in sperm count with up to 99 percent seeing sperm counts drop to zero during the treatment. More sperm is defective and malfunctioning with some having two heads or not pursuing an egg.

A class of *synthetic* chemicals known as phthalates are being blamed for the decline. These are found everywhere in plastics, shampoos, cosmetics, furniture, flame retardants, personal care products, pesticides, canned foods and even receipts. Why till receipts? Everyone touches them. Let no one delude themselves that all this is not systematic to advance the long-time agenda for human body transformation. Phthalates mimic hormones and disrupt the hormone balance causing testosterone to fall and genital birth defects in male infants. Animals and fish have been affected in the same way due to phthalates and other toxins in rivers. When fish turn gay or change sex through chemicals in rivers and streams it is a pointer to why there has been such an increase in gay people and the sexually confused. It doesn't matter to me what sexuality people choose to be, but if it's being affected by chemical pollution and consumption then we need to know. Does anyone really think that this is not connected to the transgender agenda, the war on men and the condemnation of male 'toxic masculinity'? You watch this being followed by 'toxic femininity'. It's already happening. When breastfeeding becomes 'chest-feeding', pregnant women become pregnant people along with all the other Woke claptrap you know that the world is going insane and there's a Cult scam in progress. Transgender activists are promoting the Cult agenda while Cult

billionaires support and fund the insanity as they laugh themselves to sleep at the sheer stupidity for which humans must be infamous in galaxies far, far away.

'Covid vaccines' and female infertility

We can now see why the 'vaccine' has been connected to potential infertility in women. Dr Michael Yeadon, former Vice President and Chief Scientific Advisor at Pfizer, and Dr Wolfgang Wodarg in Germany, filed a petition with the European Medicines Agency in December, 2020, urging them to stop trials for the Pfizer/BioNTech shot and all other mRNA trials until further studies had been done. They were particularly concerned about possible effects on fertility with 'vaccine'-produced antibodies attacking the protein Syncytin-1 which is responsible for developing the placenta. The result would be infertility 'of indefinite duration' in women who have the 'vaccine' with the placenta failing to form. Section 10.4.2 of the Pfizer/BioNTech trial protocol says that pregnant women or those who might become so should not have mRNA shots. Section 10.4 warns men taking mRNA shots to 'be abstinent from heterosexual intercourse' and not to donate sperm. The UK government said that it *did not know* if the mRNA procedure had an effect on fertility. *Did not know?* These people have to go to jail. UK government advice did not recommend at the start that pregnant women had the shot and said they should avoid pregnancy for at least two months after 'vaccination'. The 'advice' was later updated to pregnant women should only have the 'vaccine' if the benefits outweighed the risks to mother and foetus. What the hell is that supposed to mean? Then 'spontaneous abortions' began to appear and rapidly increase on the adverse reaction reporting schemes which include only a fraction of adverse reactions. Thousands and ever-growing numbers of 'vaccinated' women are describing changes to their menstrual cycle with heavier blood flow, irregular periods and menstruating again after going through the menopause – all links to reproduction effects. Women are passing blood clots and the lining of their uterus while men report erectile dysfunction and blood effects. Most

significantly of all *un*vaccinated women began to report similar menstrual changes after interaction with *'vaccinated'* people and men and children were also affected with bleeding noses, blood clots and other conditions. 'Shedding' is when vaccinated people can emit the content of a vaccine to affect the unvaccinated, but this is different. 'Vaccinated' people were not shedding a 'live virus' allegedly in 'vaccines' as before because the fake 'Covid vaccines' involve synthetic material and other toxicity. Doctors exposing what is happening prefer the term 'transmission' to shedding. Somehow those that have had the shots are transmitting effects to those that haven't. Dr Carrie Madej said the nano-content of the 'vaccines' can 'act like an antenna' to others around them which fits perfectly with my own conclusions. This 'vaccine' transmission phenomenon was becoming known as the book went into production and I deal with this further in the Postscript.

Vaccine effects on sterility are well known. The World Health Organization was accused in 2014 of sterilising millions of women in Kenya with the evidence confirmed by the content of the vaccines involved. The same WHO behind the 'Covid' hoax admitted its involvement for more than ten years with the vaccine programme. Other countries made similar claims. Charges were lodged by Tanzania, Nicaragua, Mexico, and the Philippines. The Gardasil vaccine claimed to protect against a genital 'virus' known as HPV has also been linked to infertility. Big Pharma and the WHO (same thing) are criminal and satanic entities. Then there's the Bill Gates Foundation which is connected through funding and shared interests with 20 pharmaceutical giants and laboratories. He stands accused of directing the policy of United Nations Children's Fund (UNICEF), vaccine alliance GAVI, and other groupings, to advance the vaccine agenda and silence opposition at great cost to women and children. At the same time Gates wants to reduce the global population. Coincidence?

Great Reset = Smart Grid = new human

The Cult agenda I have been exposing for 30 years is now being openly promoted by Cult assets like Gates and Klaus Schwab of the World Economic Forum under code-terms like the 'Great Reset', 'Build Back Better' and 'a rare but narrow window of opportunity to reflect, reimagine, and reset our world'. What provided this 'rare but narrow window of opportunity'? The 'Covid' hoax did. Who created that? *They* did. My books from not that long ago warned about the planned 'Internet of Things' (IoT) and its implications for human freedom. This was the plan to connect all technology to the Internet and artificial intelligence and today we are way down that road with an estimated 36 billion devices connected to the World Wide Web and that figure is projected to be 76 billion by 2025. I further warned that the Cult planned to go beyond that to the Internet of *Everything* when the human brain was connected via AI to the Internet and Kurzweil's 'cloud'. Now we have Cult operatives like Schwab calling for precisely that under the term 'Internet of Bodies', a fusion of the physical, digital and biological into one centrally-controlled Smart Grid system which the Cult refers to as the 'Fourth Industrial Revolution'. They talk about the 'biological', but they really mean the synthetic-biological which is required to fully integrate the human body and brain into the Smart Grid and artificial intelligence planned to replace the human mind. We have everything being synthetically manipulated including the natural world through GMO and smart dust, the food we eat and the human body itself with synthetic 'vaccines'. I said in *The Answer* that we would see the Cult push for synthetic meat to replace animals and in February, 2021, the so predictable psychopath Bill Gates called for the introduction of synthetic meat to save us all from 'climate change'. The climate hoax just keeps on giving like the 'Covid' hoax. The war on meat by vegan activists is a carbon (oops, sorry) copy of the manipulation of transgender activists. They have no idea (except their inner core) that they are being used to promote and impose the agenda of the Cult or that they are only the *vehicle* and not the *reason*. This is not to say those who choose not to eat meat shouldn't be respected and supported in that right, but there are ulterior motives

for those in power. A *Forbes* article in December, 2019, highlighted the plan so beloved of Schwab and the Cult under the heading: 'What Is The Internet of Bodies? And How Is It Changing Our World?' The article said the human body is the latest data platform (remember 'our vaccine is an operating system'). *Forbes* described the plan very accurately and the words could have come straight out of my books from long before:

The Internet of Bodies (IoB) is an extension of the IoT and basically connects the human body to a network through devices that are ingested, implanted, or connected to the body in some way. Once connected, data can be exchanged, and the body and device can be remotely monitored and controlled.

They were really describing a human hive mind with human perception centrally-dictated via an AI connection as well as allowing people to be 'remotely monitored and controlled'. Everything from a fridge to a human mind could be directed from a central point by these insane psychopaths and 'Covid vaccines' are crucial to this. Forbes explained the process I mentioned earlier of holdable and wearable technology followed by implantable. The article said there were three generations of the Internet of Bodies that include:

- Body external: These are wearable devices such as Apple Watches or Fitbits that can monitor our health.

- Body internal: These include pacemakers, cochlear implants, and digital pills that go inside our bodies to monitor or control various aspects of health.

- Body embedded: The third generation of the Internet of Bodies is embedded technology where technology and the human body are melded together and have a real-time connection to a remote machine.

Forbes noted the development of the Brain Computer Interface (BCI) which merges the brain with an external device for monitoring and controlling in real-time. 'The ultimate goal is to help restore function to individuals with disabilities by using brain signals rather than conventional neuromuscular pathways.' Oh, do fuck off. The goal of brain interface technology is controlling human thought and emotion from the central point in a hive mind serving its masters wishes. Many people are now agreeing to be chipped to open doors without a key. You can recognise them because they'll be wearing a mask, social distancing and lining up for the 'vaccine'. The Cult plans a Great Reset money system after they have completed the demolition of the global economy in which 'money' will be exchanged through communication with body operating systems. Rand Corporation, a Cult-owned think tank, said of the Internet of Bodies or IoB:

> Internet of Bodies technologies fall under the broader IoT umbrella. But as the name suggests, IoB devices introduce an even more intimate interplay between humans and gadgets. IoB devices monitor the human body, collect health metrics and other personal information, and transmit those data over the Internet. Many devices, such as fitness trackers, are already in use … IoB devices … and those in development can track, record, and store users' whereabouts, bodily functions, and what they see, hear, and even think.

Schwab's World Economic Forum, a long-winded way of saying 'fascism' or 'the Cult', has gone full-on with the Internet of Bodies in the 'Covid' era. 'We're entering the era of the Internet of Bodies', it declared, 'collecting our physical data via a range of devices that can be implanted, swallowed or worn'. The result would be a huge amount of health-related data that could improve human wellbeing around the world, and prove crucial in fighting the 'Covid-19 pandemic'. Does anyone think these clowns care about 'human wellbeing' after the death and devastation their pandemic hoax has purposely caused? Schwab and co say we should move forward with the Internet of Bodies because 'Keeping track of symptoms could help us stop the spread of infection, and quickly detect new cases'. How wonderful, but keeping track' is all they are really bothered

about. Researchers were investigating if data gathered from smartwatches and similar devices could be used as viral infection alerts by tracking the user's heart rate and breathing. Schwab said in his 2018 book *Shaping the Future of the Fourth Industrial Revolution*:

> The lines between technologies and beings are becoming blurred and not just by the ability to create lifelike robots or synthetics. Instead it is about the ability of new technologies to literally become part of us. Technologies already influence how we understand ourselves, how we think about each other, and how we determine our realities. As the technologies … give us deeper access to parts of ourselves, we may begin to integrate digital technologies into our bodies.

You can see what the game is. Twenty-four hour control and people – if you could still call them that – would never know when something would go ping and take them out of circulation. It's the most obvious rush to a global fascist dictatorship and the complete submission of humanity and yet still so many are locked away in their Cult-induced perceptual coma and can't see it.

Smart Grid control centres

The human body is being transformed by the 'vaccines' and in other ways into a synthetic cyborg that can be attached to the global Smart Grid which would be controlled from a central point and other sub-locations of Grid manipulation. Where are these planned to be? Well, China for a start which is one of the Cult's biggest centres of operation. The technological control system and technocratic rule was incubated here to be unleashed across the world after the 'Covid' hoax came out of China in 2020. Another Smart Grid location that will surprise people new to this is Israel. I have exposed in *The Trigger* how Sabbatian technocrats, intelligence and military operatives were behind the horrors of 9/11 and not '19 Arab hijackers' who somehow manifested the ability to pilot big passenger airliners when instructors at puddle-jumping flying schools described some of them as a joke. The 9/11 attacks were made possible through control of civilian and military air computer systems and those of the White House, Pentagon and connected agencies. See *The Trigger* – it

will blow your mind. The controlling and coordinating force were the Sabbatian networks in Israel and the United States which by then had infiltrated the entire US government, military and intelligence system. The real name of the American Deep State is 'Sabbatian State'. Israel is a tiny country of only nine million people, but it is one of the global centres of cyber operations and fast catching Silicon Valley in importance to the Cult. Israel is known as the 'start-up nation' for all the cyber companies spawned there with the Sabbatian specialisation of 'cyber security' that I mentioned earlier which gives those companies access to computer systems of their clients in real time through 'backdoors' written into the coding when security software is downloaded. The Sabbatian centre of cyber operations outside Silicon Valley is the Israeli military Cyber Intelligence Unit, the biggest infrastructure project in Israel's history, headquartered in the desert-city of Beersheba and involving some 20,000 'cyber soldiers'. Here are located a literal army of Internet trolls scanning social media, forums and comment lists for anyone challenging the Cult agenda. The UK military has something similar with its 77th Brigade and associated operations. The Beersheba complex includes research and development centres for other Cult operations such as Intel, Microsoft, IBM, Google, Apple, Hewlett-Packard, Cisco Systems, Facebook and Motorola. Techcrunch.com ran an article about the Beersheba global Internet technology centre headlined 'Israel's desert city of Beersheba is turning into a cybertech oasis':

> The military's massive relocation of its prestigious technology units, the presence of multinational and local companies, a close proximity to Ben Gurion University and generous government subsidies are turning Beersheba into a major global cybertech hub. Beersheba has all of the ingredients of a vibrant security technology ecosystem, including Ben Gurion University with its graduate program in cybersecurity and Cyber Security Research Center, and the presence of companies such as EMC, Deutsche Telekom, PayPal, Oracle, IBM, and Lockheed Martin. It's also the future home of the INCB (Israeli National Cyber Bureau); offers a special income tax incentive for cyber security companies, and was the site for the relocation of the army's intelligence corps units.

Sabbatians have taken over the cyber world through the following process: They scan the schools for likely cyber talent and develop them at Ben Gurion University and their period of conscription in the Israeli Defense Forces when they are stationed at the Beersheba complex. When the cyber talented officially leave the army they are funded to start cyber companies with technology developed by themselves or given to them by the state. Much of this is stolen through backdoors of computer systems around the world with America top of the list. Others are sent off to Silicon Valley to start companies or join the major ones and so we have many major positions filled by apparently 'Jewish' but really Sabbatian operatives. Google, YouTube and Facebook are all run by 'Jewish' CEOs while Twitter is all but run by ultra-Zionist hedge-fund shark Paul Singer. At the centre of the Sabbatian global cyber web is the Israeli army's Unit 8200 which specialises in hacking into computer systems of other countries, inserting viruses, gathering information, instigating malfunction, and even taking control of them from a distance. A long list of Sabbatians involved with 9/11, Silicon Valley and Israeli cyber security companies are operatives of Unit 8200. This is not about Israel. It's about the Cult. Israel is planned to be a Smart Grid hub as with China and what is happening at Beersheba is not for the benefit of Jewish people who are treated disgustingly by the Sabbatian elite that control the country. A glance at the Nuremberg Codes will tell you that.

The story is much bigger than 'Covid', important as that is to where we are being taken. Now, though, it's time to really strap in. There's more … much more …

CHAPTER ELEVEN

Who controls the Cult?

Awake, arise or be forever fall'n
John Milton, *Paradise Lost*

I have exposed this far the level of the Cult conspiracy that operates in the world of the seen and within the global secret society and satanic network which operates in the shadows one step back from the seen. The story, however, goes much deeper than that.

The 'Covid' hoax is major part of the Cult agenda, but only part, and to grasp the biggest picture we have to expand our attention beyond the realm of human sight and into the infinity of possibility that we cannot see. It is from here, ultimately, that humanity is being manipulated into a state of total control by the force which dictates the actions of the Cult. How much of reality can we see? Next to damn all is the answer. We may appear to see all there is to see in the 'space' our eyes survey and observe, but little could be further from the truth. The human 'world' is only a tiny band of frequency that the body's visual and perceptual systems can decode into *perception* of a 'world'. According to mainstream science the electromagnetic spectrum is 0.005 percent of what exists in the Universe (Fig 10). The maximum estimate I have seen is 0.5 percent and either way it's miniscule. I say it is far, far, smaller even than 0.005 percent when you compare reality we see with the totality of reality that we don't. Now get this if you are new to such information: Visible light, the only band of frequency that we can see, is a *fraction* of the 0.005

percent (Fig 11 overleaf). Take this further and realise that our universe is one of infinite universes and that universes are only a fragment of overall reality – *infinite* reality. Then compare that with the almost infinitesimal frequency band of visible light or human sight. You see that humans are as near blind as it is possible to be without actually being so. Artist and filmmaker, Sergio Toporek, said:

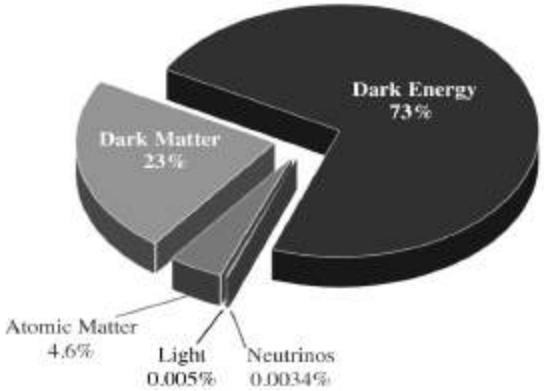

Figure 10: Humans can perceive such a tiny band of visual reality it's laughable.

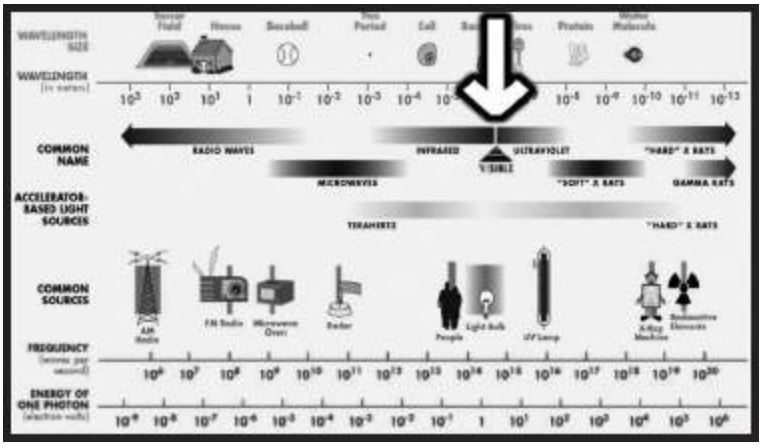

Figure 11: We can see a smear of the 0.005 percent electromagnetic spectrum, but we still know it all. Yep, makes sense.

Consider that you can see less than 1% of the electromagnetic spectrum and hear less than 1% of the acoustic spectrum. 90% of the cells in your body carry their own microbial DNA and are not 'you'. The atoms in your body are 99.9999999999999999% empty space and none of them are the ones you were born with ... Human beings have 46 chromosomes, two less than a potato.

The existence of the rainbow depends on the conical photoreceptors in your eyes; to animals without cones, the rainbow does not exist. So you don't just look at a rainbow, you create it. This is pretty amazing, especially considering that all the beautiful colours you see represent less than 1% of the electromagnetic spectrum.

Suddenly the 'world' of humans looks a very different place. Take into account, too, that Planet Earth when compared with the projected size of this single universe is the equivalent of a billionth of a pinhead. Imagine the ratio that would be when compared to infinite reality. To think that Christianity once insisted that Earth and humanity were the centre of everything. This background is vital if we are going to appreciate the nature of 'human' and how we can be manipulated by an unseen force. To human visual reality virtually *everything* is unseen and yet the prevailing perception within the institutions and so much of the public is that if we can't see it, touch it, hear it, taste it and smell it then it cannot exist. Such perception is indoctrinated and encouraged by the Cult and its agents because it isolates believers in the strictly limited, village-idiot, realm of the five senses where perceptions can be firewalled and information controlled. Most of those perpetuating the 'this-world-is-all-there-is' insanity are themselves indoctrinated into believing the same delusion. While major players and influencers know that official reality is laughable most of those in science, academia and medicine really believe the nonsense they peddle and teach succeeding generations. Those who challenge the orthodoxy are dismissed as nutters and freaks to protect the manufactured illusion from exposure. Observe the dynamic of the 'Covid' hoax and you will see how that takes the same form. The inner-circle psychopaths knows it's a gigantic scam, but almost the entirety of those imposing their fascist rules believe that 'Covid' is all that they're told it is.

Stolen identity

Ask people who they are and they will give you their name, place of birth, location, job, family background and life story. Yet that is not who they are – it is what they are *experiencing*. The difference is *absolutely crucial*. The true 'I', the eternal, infinite 'I', is consciousness,

a state of being aware. Forget 'form'. That is a vehicle for a brief experience. Consciousness does not come *from* the brain, but *through* the brain and even that is more symbolic than literal. We are awareness, pure awareness, and this is what withdraws from the body at what we call 'death' to continue our eternal beingness, *isness*, in other realms of reality within the limitlessness of infinity or the Biblical 'many mansions in my father's house'. Labels of a human life, man, woman, transgender, black, white, brown, nationality, circumstances and income are not who we are. They are what we are – awareness – is *experiencing* in a brief connection with a band of frequency we call 'human'. The labels are not the self; they are, to use the title of one of my books, a *Phantom* Self. I am not David Icke born in Leicester, England, on April 29th, 1952. I am the consciousness *having that experience*. The Cult and its non-human masters seek to convince us through the institutions of 'education', science, medicine, media and government that what we are *experiencing* is who we *are*. It's so easy to control and direct perception locked away in the bewildered illusions of the five senses with no expanded radar. Try, by contrast, doing the same with a humanity aware of its true self and its true power to consciously create its reality and experience. How is it possible to do this? We do it all day every day. If you perceive yourself as 'little me' with no power to impact upon your life and the world then your life experience will reflect that. You will hand the power you don't think you have to authority in all its forms which will use it to control your experience. This, in turn, will appear to confirm your perception of 'little me' in a self-fulfilling feedback loop. But that is what 'little me' really is – a *perception*. We are all 'big-me', infinite me, and the Cult has to make us forget that if its will is to prevail. We are therefore manipulated and pressured into self-identifying with human labels and not the consciousness/awareness *experiencing* those human labels.

The phenomenon of identity politics is a Cult-instigated manipulation technique to sub-divide previous labels into even smaller ones. A United States university employs this list of letters to

describe student identity: LGBTTQQFAGPBDSM or lesbian, gay, bisexual, transgender, transsexual, queer, questioning, flexual, asexual, gender-fuck, polyamorous, bondage/discipline, dominance/submission and sadism/masochism. I'm sure other lists are even longer by now as people feel the need to self-identity the 'I' with the minutiae of race and sexual preference. Wokers programmed by the Cult for generations believe this is about 'inclusivity' when it's really the Cult locking them away into smaller and smaller versions of Phantom Self while firewalling them from the influence of their true self, the infinite, eternal 'I'. You may notice that my philosophy which contends that we are all unique points of attention/awareness within the same infinite whole or Oneness is the ultimate non-racism. The very sense of Oneness makes the judgement of people by their body-type, colour or sexuality utterly ridiculous and confirms that racism has no understanding of reality (including anti-white racism). Yet despite my perception of life Cult agents and fast-asleep Wokers label me racist to discredit my information while they are themselves phenomenally racist and sexist. All they see is race and sexuality and they judge people as good or bad, demons or untouchables, by their race and sexuality. All they see is *Phantom Self* and perceive themselves in terms of Phantom Self. They are pawns and puppets of the Cult agenda to focus attention and self-identity in the five senses and play those identities against each other to divide and rule. Columbia University has introduced segregated graduations in another version of social distancing designed to drive people apart and teach them that different racial and cultural groups have nothing in common with each other. The last thing the Cult wants is unity. Again the pump-primers of this will be Cult operatives in the knowledge of what they are doing, but the rest are just the Phantom Self blind leading the Phantom Self blind. We *do* have something in common – we are all *the same consciousness* having different temporary experiences.

What is this 'human'?

Yes, what *is* 'human'? That is what we are supposed to be, right? I mean 'human'? True, but 'human' is the experience not the 'I'. Break it down to basics and 'human' is the way that information is processed. If we are to experience and interact with this band of frequency we call the 'world' we must have a vehicle that operates within that band of frequency. Our consciousness in its prime form cannot do that; it is way beyond the frequency of the human realm. My consciousness or awareness could not tap these keys and pick up the cup in front of me in the same way that radio station A cannot interact with radio station B when they are on different frequencies. The human body is the means through which we have that interaction. I have long described the body as a biological computer which processes information in a way that allows consciousness to experience this reality. The body is a receiver, transmitter and processor of information in a particular way that we call human. We visually perceive only the world of the five senses in a wakened state – that is the limit of the body's visual decoding system. In truth it's not even visual in the way we experience 'visual reality' as I will come to in a moment. We are 'human' because the body processes the information sources of human into a reality and behaviour system that we *perceive* as human. Why does an elephant act like an elephant and not like a human or a duck? The elephant's biological computer is a different information field and processes information according to that program into a visual and behaviour type we call an elephant. The same applies to everything in our reality. These body information fields are perpetuated through procreation (like making a copy of a software program). The Cult wants to break that cycle and intervene technologically to transform the human information field into one that will change what we call humanity. If it can change the human information field it will change the way that field processes information and change humanity both 'physically' and psychologically. Hence the *messenger* (information) RNA 'vaccines' and so much more that is targeting human genetics by changing the body's information – *messaging* – construct through food, drink, radiation, toxicity and other means.

Reality that we experience is nothing like reality as it really is in the same way that the reality people experience in virtual reality games is not the reality they are really living in. The game is only a decoded source of information that appears to be a reality. Our world is also an information construct – a *simulation* (more later). In its base form our reality is a wavefield of information much the same in theme as Wi-Fi. The five senses decode wavefield information into electrical information which they communicate to the brain to decode into holographic (illusory 'physical') information. Different parts of the brain specialise in decoding different senses and the information is fused into a reality that appears to be outside of us but is really inside the brain and the genetic structure in general (Fig 12 overleaf). DNA is a receiver-transmitter of information and a vital part of this decoding process and the body's connection to other realities. Change DNA and you change the way we decode and connect with reality – see 'Covid vaccines'. Think of computers decoding Wi-Fi. You have information encoded in a radiation field and the computer decodes that information into a very different form on the screen. You can't see the Wi-Fi until its information is made manifest on the screen and the information on the screen is inside the computer and not outside. I have just described how we decode the 'human world'. All five senses decode the waveform 'Wi-Fi' field into electrical signals and the brain (computer) constructs reality inside the brain and not outside – 'You don't just look at a rainbow, you create it'. Sound is a simple example. We don't hear sound until the brain decodes it. Waveform sound waves are picked up by the hearing sense and communicated to the brain in an electrical form to be decoded into the sounds that we hear. Everything we hear is inside the brain along with everything we see, feel, smell and taste. Words and language are waveform fields generated by our vocal chords which pass through this process until they are decoded by the brain into words that we hear. Different languages are different frequency fields or sound waves generated by vocal chords. Late British philosopher Alan Watts said:

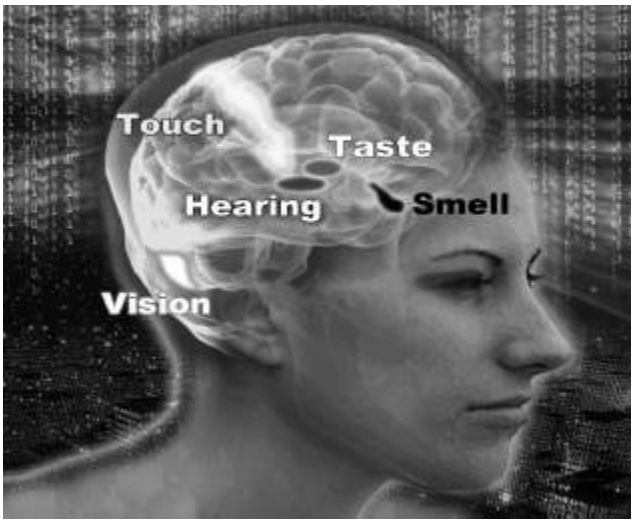

Figure 12: The brain receives information from the five senses and constructs from that our perceived reality.

[Without the brain] the world is devoid of light, heat, weight, solidity, motion, space, time or any other imaginable feature. All these phenomena are interactions, or transactions, of vibrations with a certain arrangement of neurons.

That's exactly what they are and scientist Robert Lanza describes in his book, *Biocentrism*, how we decode electromagnetic waves and energy into visual and 'physical' experience. He uses the example of a flame emitting photons, electromagnetic energy, each pulsing electrically and magnetically:

… these … invisible electromagnetic waves strike a human retina, and if (and only if) the waves happen to measure between 400 and 700 nano meters in length from crest to crest, then their energy is just right to deliver a stimulus to the 8 million cone-shaped cells in the retina.

Each in turn send an electrical pulse to a neighbour neuron, and on up the line this goes, at 250 mph, until it reaches the … occipital lobe of the brain, in the back of the head. There, a cascading complex of neurons fire from the incoming stimuli, and we subjectively perceive this experience as a yellow brightness occurring in a place we have been conditioned to call the 'external world'.

You hear what you decode

If a tree falls or a building collapses they make no noise unless someone is there to decode the energetic waves generated by the disturbance into what we call sound. Does a falling tree make a noise? Only if you hear it – *decode* it. Everything in our reality is a frequency field of information operating within the overall 'Wi-Fi' field that I call The Field. A vibrational disturbance is generated in The Field by the fields of the falling tree or building. These disturbance waves are what we decode into the sound of them falling. If no one is there to do that then neither will make any noise. Reality is created by the observer – *decoder* – and the *perceptions* of the observer affect the decoding process. For this reason different people – different *perceptions* – will perceive the same reality or situation in a different way. What one may perceive as a nightmare another will see as an opportunity. The question of why the Cult is so focused on controlling human perception now answers itself. All experienced reality is the act of decoding and we don't experience Wi-Fi until it is decoded on the computer screen. The sight and sound of an Internet video is encoded in the Wi-Fi all around us, but we don't see or hear it until the computer decodes that information. Taste, smell and touch are all phenomena of the brain as a result of the same process. We don't taste, smell or feel anything except in the brain and there are pain relief techniques that seek to block the signal from the site of discomfort to the brain because if the brain doesn't decode that signal we don't feel pain. Pain is in the brain and only appears to be at the point of impact thanks to the feedback loop between them. We don't see anything until electrical information from the sight senses is decoded in an area at the back of the brain. If that area is damaged we can go blind when our eyes are perfectly okay. So why do we go blind if we damage an eye? We damage the information processing between the waveform visual information and the visual decoding area of the brain. If information doesn't reach the brain in a form it can decode then we can't see the visual reality that it represents. What's more the brain is decoding only a fraction of the information it receives and the rest is absorbed by the

sub-conscious mind. This explanation is from the science magazine, *Wonderpedia*:

> Every second, 11 million sensations crackle along these [brain] pathways ... The brain is confronted with an alarming array of images, sounds and smells which it rigorously filters down until it is left with a manageable list of around 40. Thus 40 sensations per second make up what we perceive as reality.

The 'world' is not what people are told to believe that is it and the inner circles of the Cult *know that*.

Illusory 'physical' reality

We can only see a smear of 0.005 percent of the Universe which is only one of a vast array of universes – 'mansions' – within infinite reality. Even then the brain decodes only 40 pieces of information ('sensations') from a potential *11 million* that we receive every second. Two points strike you from this immediately: The sheer breathtaking stupidity of believing we know anything so rigidly that there's nothing more to know; and the potential for these processes to be manipulated by a malevolent force to control the reality of the population. One thing I can say for sure with no risk of contradiction is that when you can perceive an almost indescribable fraction of infinite reality there is always more to know as in tidal waves of it. Ancient Greek philosopher Socrates was so right when he said that wisdom is to know how little we know. How obviously true that is when you think that we are experiencing a physical world of solidity that is neither physical nor solid and a world of apartness when everything is connected. Cult-controlled 'science' dismisses the so-called 'paranormal' and all phenomena related to that when the 'para'-normal is perfectly normal and explains the alleged 'great mysteries' which dumbfound scientific minds. There is a reason for this. A 'scientific mind' in terms of the mainstream is a material mind, a five-sense mind imprisoned in see it, touch it, hear it, smell it and taste it. Phenomena and happenings that can't be explained that way leave the 'scientific mind' bewildered and the rule is that if they

can't account for why something is happening then it can't, by definition, be happening. I beg to differ. Telepathy is thought waves passing through The Field (think wave disturbance again) to be decoded by someone able to connect with that wavelength (information). For example: You can pick up the thought waves of a friend at any distance and at the very least that will bring them to mind. A few minutes later the friend calls you. 'My god', you say, 'that's incredible – I was just thinking of you.' Ah, but *they* were thinking of *you* before they made the call and that's what you decoded. Native peoples not entrapped in five-sense reality do this so well it became known as the 'bush telegraph'. Those known as psychics and mediums (genuine ones) are doing the same only across dimensions of reality. 'Mind over matter' comes from the fact that matter and mind are the *same*. The state of one influences the state of the other. Indeed one *and* the other are illusions. They are aspects of the same field. Paranormal phenomena are all explainable so why are they still considered 'mysteries' or not happening? Once you go down this road of understanding you begin to expand awareness beyond the five senses and that's the nightmare for the Cult.

Figure 13: Holograms are not solid, but the best ones appear to be.

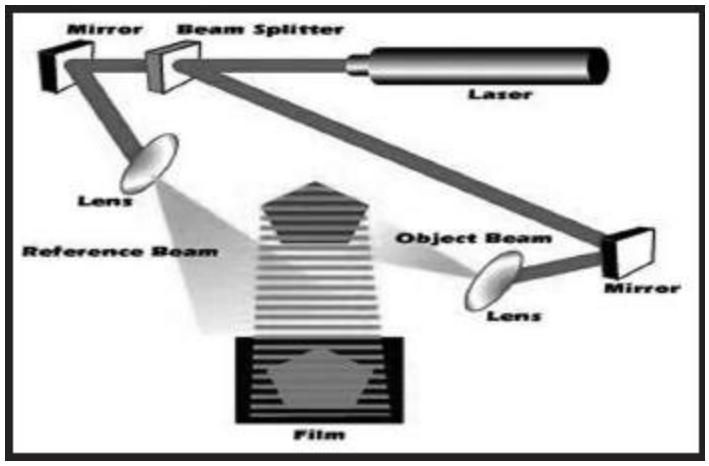

Figure 14: How holograms are created by capturing a waveform version of the subject image.

Holographic 'solidity'

Our reality is not solid, it is holographic. We are now well aware of holograms which are widely used today. Two-dimensional information is decoded into a three-dimensional reality that is not solid although can very much appear to be (Fig 13). Holograms are created with a laser divided into two parts. One goes directly onto a holographic photographic print ('reference beam') and the other takes a waveform image of the subject ('working beam') before being directed onto the print where it 'collides' with the other half of the laser (Fig 14). This creates a *waveform* interference pattern which contains the wavefield information of whatever is being photographed (Fig 15 overleaf). The process can be likened to dropping pebbles in a pond. Waves generated by each one spread out across the water to collide with the others and create a wave representation of where the stones fell and at what speed, weight and distance. A waveform interference pattern of a hologram is akin to the waveform information in The Field which the five senses decode into electrical signals to be decoded by the brain into a holographic illusory 'physical' reality. In the same way when a laser (think human attention) is directed at the waveform interference pattern a three-dimensional version of the subject is projected into apparently 'solid' reality (Fig 16). An amazing trait of holograms reveals more 'paranormal mysteries'. Information of the *whole*

hologram is encoded in waveform in every part of the interference pattern by the way they are created. This means that every *part* of a hologram is a smaller version of the whole. Cut the interference wave-pattern into four and you won't get four parts of the image. You get quarter-sized versions of the *whole* image. The body is a hologram and the same applies. Here we have the basis of acupuncture, reflexology and other forms of healing which identify representations of the whole body in all of the parts, hands, feet, ears, everywhere. Skilled palm readers can do what they do because the information of whole body is encoded in the hand. The concept of as above, so below, comes from this.

Figure 15: A waveform interference pattern that holds the information that transforms into a hologram.

Figure 16: Holographic people including 'Elvis' holographically inserted to sing a duet with Celine Dion.

The question will be asked of why, if solidity is illusory, we can't just walk through walls and each other. The resistance is not solid against solid; it is electromagnetic field against electromagnetic field and we decode this into the *experience* of solid against solid. We should also not underestimate the power of belief to dictate reality. What you believe is impossible *will be*. Your belief impacts on your decoding processes and they won't decode what you think is impossible. What we believe we perceive and what we perceive we experience. 'Can't dos' and 'impossibles' are like a firewall in a computer system that won't put on the screen what the firewall blocks. How vital that is to understanding how human experience has been hijacked. I explain in *The Answer*, *Everything You Need To Know But Have Never Been Told* and other books a long list of 'mysteries' and 'paranormal' phenomena that are not mysterious and perfectly normal once you realise what reality is and how it works. 'Ghosts' can be seen to pass through 'solid' walls because the walls are not solid and the ghost is a discarnate entity operating on a frequency so different to that of the wall that it's like two radio stations sharing the same space while never interfering with each other. I have seen ghosts do this myself. The apartness of people and objects is also an illusion. Everything is connected by the Field like all sea life is connected by the sea. It's just that within the limits of our visual reality we only 'see' holographic information and not the field of information that connects everything and from which the holographic world is made manifest. If you can only see holographic 'objects' and not the field that connects them they will appear to you as unconnected to each other in the same way that we see the computer while not seeing the Wi-Fi.

What you don't know *can* hurt you

Okay, we return to those 'two worlds' of human society and the Cult with its global network of interconnecting secret societies and satanic groups which manipulate through governments, corporations, media, religions, etc. The fundamental difference between them is *knowledge*. The idea has been to keep humanity

ignorant of the plan for its total enslavement underpinned by a crucial ignorance of reality – who we are and where we are – and how we interact with it. 'Human' should be the interaction between our expanded eternal consciousness and the five-sense body experience. We are meant to be *in* this world in terms of the five senses but not *of* this world in relation to our greater consciousness and perspective. In that state we experience the small picture of the five senses within the wider context of the big picture of awareness beyond the five senses. Put another way the five senses see the dots and expanded awareness connects them into pictures and patterns that give context to the apparently random and unconnected. Without the context of expanded awareness the five senses see only apartness and randomness with apparently no meaning. The Cult and its other-dimensional controllers seek to intervene in the frequency realm where five-sense reality is supposed to connect with expanded reality and to keep the two apart (more on this in the final chapter). When that happens five-sense mental and emotional processes are no longer influenced by expanded awareness, or the True 'I', and instead are driven by the isolated perceptions of the body's decoding systems. They are in the world *and* of it. Here we have the human plight and why humanity with its potential for infinite awareness can be so easily manipulatable and descend into such extremes of stupidity.

Once the Cult isolates five-sense mind from expanded awareness it can then program the mind with perceptions and beliefs by controlling information that the mind receives through the 'education' system of the formative years and the media perceptual bombardment and censorship of an entire lifetime. Limit perception and a sense of the possible through limiting knowledge by limiting and skewing information while censoring and discrediting that which could set people free. As the title of another of my books says *… And The Truth Shall Set You Free*. For this reason the last thing the Cult wants in circulation is the truth about anything – especially the reality of the eternal 'I' – and that's why it is desperate to control information. The Cult knows that information becomes perception

which becomes behaviour which, collectively, becomes human society. Cult-controlled and funded mainstream 'science' denies the existence of an eternal 'I' and seeks to dismiss and trash all evidence to the contrary. Cult-controlled mainstream religion has a version of 'God' that is little more than a system of control and dictatorship that employs threats of damnation in an afterlife to control perceptions and behaviour in the here and now through fear and guilt. Neither is true and it's the 'neither' that the Cult wishes to suppress. This 'neither' is that everything is an expression, a point of attention, within an infinite state of consciousness which is the real meaning of the term 'God'.

Perceptual obsession with the 'physical body' and five-senses means that 'God' becomes personified as a bearded bloke sitting among the clouds or a raging bully who loves us if we do what 'he' wants and condemns us to the fires of hell if we don't. These are no more than a 'spiritual' fairy tales to control and dictate events and behaviour through fear of this 'God' which has bizarrely made 'God-fearing' in religious circles a state to be desired. I would suggest that fearing *anything* is not to be encouraged and celebrated, but rather deleted. You can see why 'God fearing' is so beneficial to the Cult and its religions when *they* decide what 'God' wants and what 'God' demands (the Cult demands) that everyone do. As the great American comedian Bill Hicks said satirising a Christian zealot: 'I think what God meant to say.' How much of this infinite awareness ('God') that we access is decided by how far we choose to expand our perceptions, self-identity and sense of the possible. The scale of self-identity reflects itself in the scale of awareness that we can connect with and are influenced by – how much knowing and insight we have instead of programmed perception. You cannot expand your awareness into the infinity of possibility when you believe that you are little me Peter the postman or Mary in marketing and nothing more. I'll deal with this in the concluding chapter because it's crucial to how we turnaround current events.

Where the Cult came from

When I realised in the early 1990s there was a Cult network behind global events I asked the obvious question: When did it start? I took it back to ancient Rome and Egypt and on to Babylon and Sumer in Mesopotamia, the 'Land Between Two Rivers', in what we now call Iraq. The two rivers are the Tigris and Euphrates and this region is of immense historical and other importance to the Cult, as is the land called Israel only 550 miles away by air. There is much more going with deep esoteric meaning across this whole region. It's not only about 'wars for oil'. Priceless artefacts from Mesopotamia were stolen or destroyed after the American and British invasion of Iraq in 2003 justified by the lies of Boy Bush and Tony Blair (their Cult masters) about non-existent 'weapons of mass destruction'. Mesopotamia was the location of Sumer (about 5,400BC to 1,750BC), and Babylon (about 2,350BC to 539BC). Sabbatians may have become immensely influential in the Cult in modern times but they are part of a network that goes back into the mists of history. Sumer is said by historians to be the 'cradle of civilisation'. I disagree. I say it was the re-start of what we call human civilisation after cataclysmic events symbolised in part as the 'Great Flood' destroyed the world that existed before. These fantastic upheavals that I have been describing in detail in the books since the early1990s appear in accounts and legends of ancient cultures across the world and they are supported by geological and biological evidence. Stone tablets found in Iraq detailing the Sumer period say the cataclysms were caused by non-human 'gods' they call the Anunnaki. These are described in terms of extraterrestrial visitations in which knowledge supplied by the Anunnaki is said to have been the source of at least one of the world's oldest writing systems and developments in astronomy, mathematics and architecture that were way ahead of their time. I have covered this subject at length in *The Biggest Secret* and *Children of the Matrix* and the same basic 'Anunnaki' story can be found in Zulu accounts in South Africa where the late and very great Zulu high shaman Credo Mutwa told me that the Sumerian Anunnaki were known by Zulus as the Chitauri or 'children of the serpent'. See my six-hour video interview with Credo on this subject entitled *The*

Reptilian Agenda recorded at his then home near Johannesburg in 1999 which you can watch on the Ickonic media platform.

The Cult emerged out of Sumer, Babylon and Egypt (and elsewhere) and established the Roman Empire before expanding with the Romans into northern Europe from where many empires were savagely imposed in the form of Cult-controlled societies all over the world. Mass death and destruction was their calling card. The Cult established its centre of operations in Europe and European Empires were Cult empires which allowed it to expand into a global force. Spanish and Portuguese colonialists headed for Central and South America while the British and French targeted North America. Africa was colonised by Britain, France, Belgium, the Netherlands, Portugal, Spain, Italy, and Germany. Some like Britain and France moved in on the Middle East. The British Empire was by far the biggest for a simple reason. By now Britain was the headquarters of the Cult from which it expanded to form Canada, the United States, Australia and New Zealand. The Sun never set on the British Empire such was the scale of its occupation. London remains a global centre for the Cult along with Rome and the Vatican although others have emerged in Israel and China. It is no accident that the 'virus' is alleged to have come out of China while Italy was chosen as the means to terrify the Western population into compliance with 'Covid' fascism. Nor that Israel has led the world in 'Covid' fascism and mass 'vaccination'.

You would think that I would mention the United States here, but while it has been an important means of imposing the Cult's will it is less significant than would appear and is currently in the process of having what power it does have deleted. The Cult in Europe has mostly loaded the guns for the US to fire. America has been controlled from Europe from the start through Cult operatives in Britain and Europe. The American Revolution was an illusion to make it appear that America was governing itself while very different forces were pulling the strings in the form of Cult families such as the Rothschilds through the Rockefellers and other subordinates. The Rockefellers are extremely close to Bill Gates and

established both scalpel and drug 'medicine' and the World Health Organization. They play a major role in the development and circulation of vaccines through the Rockefeller Foundation on which Bill Gates said his Foundation is based. Why wouldn't this be the case when the Rockefellers and Gates are on the same team? Cult infiltration of human society goes way back into what we call history and has been constantly expanding and centralising power with the goal of establishing a global structure to dictate everything. Look how this has been advanced in great leaps with the 'Covid' hoax.

The non-human dimension

I researched and observed the comings and goings of Cult operatives through the centuries and even thousands of years as they were born, worked to promote the agenda within the secret society and satanic networks, and then died for others to replace them. Clearly there had to be a coordinating force that spanned this entire period while operatives who would not have seen the end goal in their lifetimes came and went advancing the plan over millennia. I went in search of that coordinating force with the usual support from the extraordinary synchronicity of my life which has been an almost daily experience since 1990. I saw common themes in religious texts and ancient cultures about a non-human force manipulating human society from the hidden. Christianity calls this force Satan, the Devil and demons; Islam refers to the Jinn or Djinn; Zulus have their Chitauri (spelt in other ways in different parts of Africa); and the Gnostic people in Egypt in the period around and before 400AD referred to this phenomena as the 'Archons', a word meaning rulers in Greek. Central American cultures speak of the 'Predators' among other names and the same theme is everywhere. I will use 'Archons' as a collective name for all of them. When you see how their nature and behaviour is described all these different sources are clearly talking about the same force. Gnostics described the Archons in terms of 'luminous fire' while Islam relates the Jinn to 'smokeless fire'. Some refer to beings in form that could occasionally be seen, but the most common of common theme is that they operate from

unseen realms which means almost all existence to the visual processes of humans. I had concluded that this was indeed the foundation of human control and that the Cult was operating within the human frequency band on behalf of this hidden force when I came across the writings of Gnostics which supported my conclusions in the most extraordinary way.

A sealed earthen jar was found in 1945 near the town of Nag Hammadi about 75-80 miles north of Luxor on the banks of the River Nile in Egypt. Inside was a treasure trove of manuscripts and texts left by the Gnostic people some 1,600 years earlier. They included 13 leather-bound papyrus codices (manuscripts) and more than 50 texts written in Coptic Egyptian estimated to have been hidden in the jar in the period of 400AD although the source of the information goes back much further. Gnostics oversaw the Great or Royal Library of Alexandria, the fantastic depository of ancient texts detailing advanced knowledge and accounts of human history. The Library was dismantled and destroyed in stages over a long period with the death-blow delivered by the Cult-established Roman Church in the period around 415AD. The Church of Rome was the Church of Babylon relocated as I said earlier. Gnostics were not a race. They were a way of perceiving reality. Whenever they established themselves and their information circulated the terrorists of the Church of Rome would target them for destruction. This happened with the Great Library and with the Gnostic Cathars who were burned to death by the psychopaths after a long period of oppression at the siege of the Castle of Monségur in southern France in 1244. The Church has always been terrified of Gnostic information which demolishes the official Christian narrative although there is much in the Bible that supports the Gnostic view if you read it in another way. To anyone studying the texts of what became known as the Nag Hammadi Library it is clear that great swathes of Christian and Biblical belief has its origin with Gnostics sources going back to Sumer. Gnostic themes have been twisted to manipulate the perceived reality of Bible believers. Biblical texts have been in the open for centuries where they could be changed while Gnostic

documents found at Nag Hammadi were sealed away and untouched for 1,600 years. What you see is what they wrote.

Use your *pneuma* not your *nous*

Gnosticism and Gnostic come from 'gnosis' which means knowledge, or rather *secret* knowledge, in the sense of spiritual awareness – knowledge about reality and life itself. The desperation of the Cult's Church of Rome to destroy the Gnostics can be understood when the knowledge they were circulating was the last thing the Cult wanted the population to know. Sixteen hundred years later the same Cult is working hard to undermine and silence me for the same reason. The dynamic between knowledge and ignorance is a constant. 'Time' appears to move on, but essential themes remain the same. We are told to 'use your nous', a Gnostic word for head/brain/intelligence. They said, however, that spiritual awakening or 'salvation' could only be secured by expanding awareness *beyond* what they called *nous* and into *pneuma* or Infinite Self. Obviously as I read these texts the parallels with what I have been saying since 1990 were fascinating to me. There is a universal truth that spans human history and in that case why wouldn't we be talking the same language 16 centuries apart? When you free yourself from the perception program of the five senses and explore expanded realms of consciousness you are going to connect with the same information no matter what the perceived 'era' within a manufactured timeline of a single and tiny range of manipulated frequency. Humans working with 'smart' technology or knocking rocks together in caves is only a timeline appearing to operate within the human frequency band. Expanded awareness and the knowledge it holds have always been there whether the era be Stone Age or computer age. We can only access that knowledge by opening ourselves to its frequency which the five-sense prison cell is designed to stop us doing. Gates, Fauci, Whitty, Vallance, Zuckerberg, Brin, Page, Wojcicki, Bezos, and all the others behind the 'Covid' hoax clearly have a long wait before their range of frequency can make that connection given that an open heart is

crucial to that as we shall see. Instead of accessing knowledge directly through expanded awareness it is given to Cult operatives by the secret society networks of the Cult where it has been passed on over thousands of years outside the public arena. Expanded realms of consciousness is where great artists, composers and writers find their inspiration and where truth awaits anyone open enough to connect with it. We need to go there fast.

Archon hijack

A fifth of the Nag Hammadi texts describe the existence and manipulation of the Archons led by a 'Chief Archon' they call 'Yaldabaoth', or the 'Demiurge', and this is the Christian 'Devil', 'Satan', 'Lucifer', and his demons. Archons in Biblical symbolism are the 'fallen ones' which are also referred to as fallen angels after the angels expelled from heaven according to the Abrahamic religions of Judaism, Christianity and Islam. These angels are claimed to tempt humans to 'sin' ongoing and you will see how accurate that symbolism is during the rest of the book. The theme of 'original sin' is related to the 'Fall' when Adam and Eve were 'tempted by the serpent' and fell from a state of innocence and 'obedience' (connection) with God into a state of disobedience (disconnection). The Fall is said to have brought sin into the world and corrupted everything including human nature. Yaldabaoth, the 'Lord Archon', is described by Gnostics as a 'counterfeit spirit', 'The Blind One', 'The Blind God', and 'The Foolish One'. The Jewish name for Yaldabaoth in Talmudic writings is Samael which translates as 'Poison of God', or 'Blindness of God'. You see the parallels. Yaldabaoth in Islamic belief is the Muslim Jinn devil known as Shaytan – Shaytan is Satan as the same themes are found all over the world in every religion and culture. The 'Lord God' of the Old Testament is the 'Lord Archon' of Gnostic manuscripts and that's why he's such a bloodthirsty bastard. Satan is known by Christians as 'the Demon of Demons' and Gnostics called Yaldabaoth the 'Archon of Archons'. Both are known as 'The Deceiver'. We are talking about the same 'bloke' for sure and these common themes

using different names, storylines and symbolism tell a common tale of the human plight.

Archons are referred to in Nag Hammadi documents as mind parasites, inverters, guards, gatekeepers, detainers, judges, pitiless ones and deceivers. The 'Covid' hoax alone is a glaring example of all these things. The Biblical 'God' is so different in the Old and New Testaments because they are not describing the same phenomenon. The vindictive, angry, hate-filled, 'God' of the Old Testament, known as Yahweh, is Yaldabaoth who is depicted in Cult-dictated popular culture as the 'Dark Lord', 'Lord of Time', Lord (Darth) Vader and Dormammu, the evil ruler of the 'Dark Dimension' trying to take over the 'Earth Dimension' in the Marvel comic movie, *Dr Strange*. Yaldabaoth is both the Old Testament 'god' and the Biblical 'Satan'. Gnostics referred to Yaldabaoth as the 'Great Architect of the Universe'and the Cult-controlled Freemason network calls their god 'the 'Great Architect of the Universe' (also Grand Architect). The 'Great Architect' Yaldabaoth is symbolised by the Cult as the all-seeing eye at the top of the pyramid on the Great Seal of the United States and the dollar bill. Archon is encoded in *arch*-itect as it is in *arch*-angels and *arch*-bishops. All religions have the theme of a force for good and force for evil in some sort of spiritual war and there is a reason for that – the theme is true. The Cult and its non-human masters are quite happy for this to circulate. They present themselves as the force for good fighting evil when they are really the force of evil (absence of love). The whole foundation of Cult modus operandi is inversion. They promote themselves as a force for good and anyone challenging them in pursuit of peace, love, fairness, truth and justice is condemned as a satanic force for evil. This has been the game plan throughout history whether the Church of Rome inquisitions of non-believers or 'conspiracy theorists' and 'anti-vaxxers' of today. The technique is the same whatever the timeline era.

Yaldabaoth is revolting (true)

Yaldabaoth and the Archons are said to have revolted against God with Yaldabaoth claiming to *be* God – the *All That Is*. The Old Testament 'God' (Yaldabaoth) demanded to be worshipped as such: ' *I am* the LORD, and there is none else, there is no God beside me' (Isaiah 45:5). I have quoted in other books a man who said he was the unofficial son of the late Baron Philippe de Rothschild of the Mouton-Rothschild wine producing estates in France who died in 1988 and he told me about the Rothschild 'revolt from God'. The man said he was given the name Phillip Eugene de Rothschild and we shared long correspondence many years ago while he was living under another identity. He said that he was conceived through 'occult incest' which (within the Cult) was 'normal and to be admired'. 'Phillip' told me about his experience attending satanic rituals with rich and famous people whom he names and you can see them and the wider background to Cult Satanism in my other books starting with *The Biggest Secret*. Cult rituals are interactions with Archontic 'gods'. 'Phillip' described Baron Philippe de Rothschild as 'a master Satanist and hater of God' and he used the same term 'revolt from God' associated with Yaldabaoth/Satan/Lucifer/the Devil in describing the Sabbatian Rothschild dynasty. 'I played a key role in my family's revolt from God', he said. That role was to infiltrate in classic Sabbatian style the Christian Church, but eventually he escaped the mind-prison to live another life. The Cult has been targeting religion in a plan to make worship of the Archons the global one-world religion. Infiltration of Satanism into modern 'culture', especially among the young, through music videos, stage shows and other means, is all part of this.

Nag Hammadi texts describe Yaldabaoth and the Archons in their prime form as energy – consciousness – and say they can take form if they choose in the same way that consciousness takes form as a human. Yaldabaoth is called 'formless' and represents a deeply inverted, distorted and chaotic state of consciousness which seeks to attached to humans and turn them into a likeness of itself in an attempt at assimilation. For that to happen it has to manipulate

humans into low frequency mental and emotional states that match its own. Archons can certainly appear in human form and this is the origin of the psychopathic personality. The energetic distortion Gnostics called Yaldabaoth *is* psychopathy. When psychopathic Archons take human form that human will be a psychopath as an expression of Yaldabaoth consciousness. Cult psychopaths are Archons in human form. The principle is the same as that portrayed in the 2009 *Avatar* movie when the American military travelled to a fictional Earth-like moon called Pandora in the Alpha Centauri star system to infiltrate a society of blue people, or Na'vi, by hiding within bodies that looked like the Na'vi. Archons posing as humans have a particular hybrid information field, part human, part Archon, (the ancient 'demigods') which processes information in a way that manifests behaviour to match their psychopathic evil, lack of empathy and compassion, and stops them being influenced by the empathy, compassion and love that a fully-human information field is capable of expressing. Cult bloodlines interbreed, be they royalty or dark suits, for this reason and you have their obsession with incest. Interbreeding with full-blown humans would dilute the Archontic energy field that guarantees psychopathy in its representatives in the human realm.

Gnostic writings say the main non-human forms that Archons take are *serpentine* (what I have called for decades 'reptilian' amid unbounded ridicule from the Archontically-programmed) and what Gnostics describe as 'an unborn baby or foetus with grey skin and dark, unmoving eyes'. This is an excellent representation of the ET 'Greys' of UFO folklore which large numbers of people claim to have seen and been abducted by – Zulu shaman Credo Mutwa among them. I agree with those that believe in extraterrestrial or interdimensional visitations today and for thousands of years past. No wonder with their advanced knowledge and technological capability they were perceived and worshipped as gods for technological and other 'miracles' they appeared to perform. Imagine someone arriving in a culture disconnected from the modern world with a smartphone and computer. They would be

seen as a 'god' capable of 'miracles'. The Renegade Mind, however, wants to know the source of everything and not only the way that source manifests as human or non-human. In the same way that a Renegade Mind seeks the original source material for the 'Covid virus' to see if what is claimed is true. The original source of Archons in form is consciousness – the distorted state of consciousness known to Gnostics as Yaldabaoth.

'Revolt from God' is energetic disconnection

Where I am going next will make a lot of sense of religious texts and ancient legends relating to 'Satan', Lucifer' and the 'gods'. Gnostic descriptions sync perfectly with the themes of my own research over the years in how they describe a consciousness distortion seeking to impose itself on human consciousness. I've referred to the core of infinite awareness in previous books as Infinite Awareness in Awareness of Itself. By that I mean a level of awareness that knows that it is all awareness and is aware of all awareness. From here comes the frequency of love in its true sense and balance which is what love is on one level – the balance of all forces into a single whole called Oneness and Isness. The more we disconnect from this state of love that many call 'God' the constituent parts of that Oneness start to unravel and express themselves as a part and not a whole. They become individualised as intellect, mind, selfishness, hatred, envy, desire for power over others, and such like. This is not a problem in the greater scheme in that 'God', the *All That Is*, can experience all these possibilities through different expressions of itself including humans. What we as expressions of the whole experience the *All That Is* experiences. We are the *All That Is* experiencing itself. As we withdraw from that state of Oneness we disconnect from its influence and things can get very unpleasant and very stupid. Archontic consciousness is at the extreme end of that. It has so disconnected from the influence of Oneness that it has become an inversion of unity and love, an inversion of everything, an inversion of life itself. Evil is appropriately live written backwards. Archontic consciousness is obsessed with death, an inversion of life,

and so its manifestations in Satanism are obsessed with death. They use inverted symbols in their rituals such as the inverted pentagram and cross. Sabbatians as Archontic consciousness incarnate invert Judaism and every other religion and culture they infiltrate. They seek disunity and chaos and they fear unity and harmony as they fear love like garlic to a vampire. As a result the Cult, Archons incarnate, act with such evil, psychopathy and lack of empathy and compassion disconnected as they are from the source of love. How could Bill Gates and the rest of the Archontic psychopaths do what they have to human society in the 'Covid' era with all the death, suffering and destruction involved and have no emotional consequence for the impact on others? Now you know. Why have Zuckerberg, Brin, Page, Wojcicki and company callously censored information warning about the dangers of the 'vaccine' while thousands have been dying and having severe, sometimes life-changing reactions? Now you know. Why have Tedros, Fauci, Whitty, Vallance and their like around the world been using case and death figures they're aware are fraudulent to justify lockdowns and all the deaths and destroyed lives that have come from that? Now you know. Why did Christian Drosten produce and promote a 'testing' protocol that he knew couldn't test for infectious disease which led to a global human catastrophe. Now you know. The Archontic mind doesn't give a shit (Fig 17). I personally think that Gates and major Cult insiders are a form of AI cyborg that the Archons want humans to become.

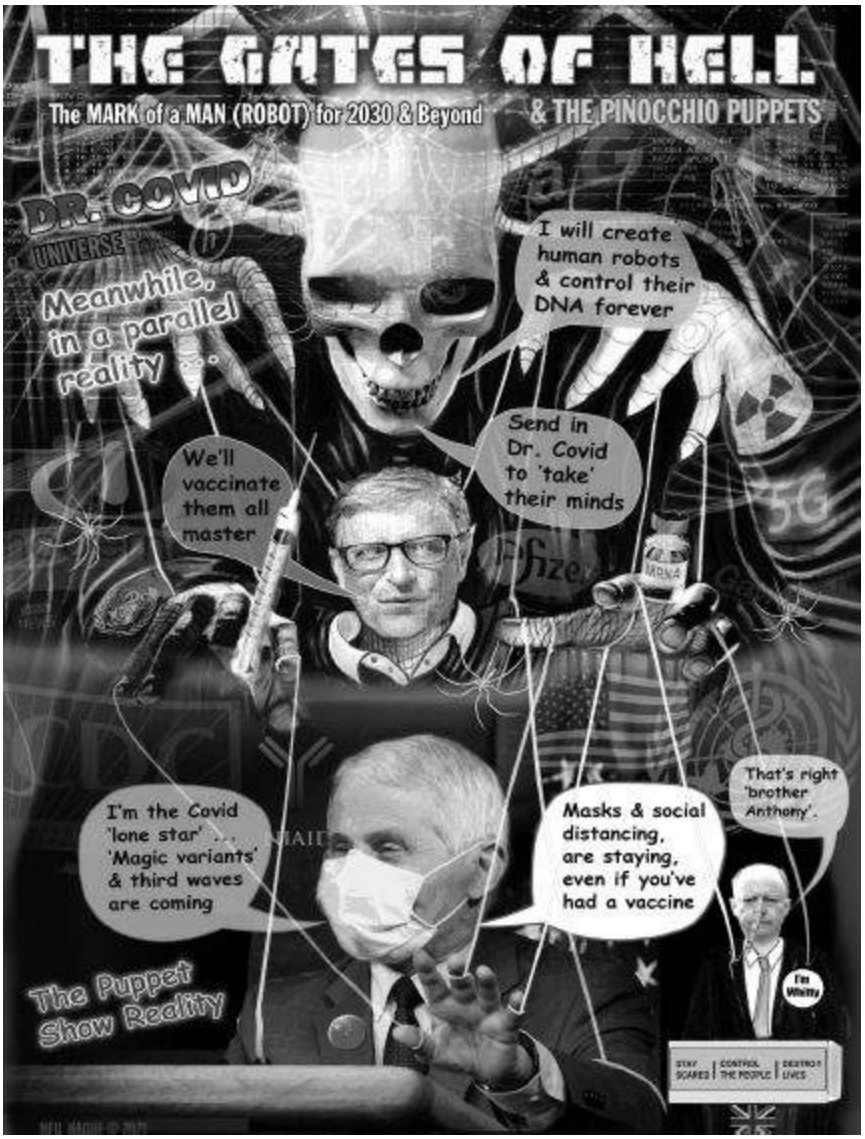
THE GATES OF HELL
The MARK of a MAN (ROBOT) for 2030 & Beyond
& THE PINOCCHIO PUPPETS
DR. COVID
UNIVERSE
Meanwhile, in a parallel reality ...
I will create human robots & control their DNA forever
Send in Dr. Covid to 'take' their minds
We'll vaccinate them all master
5G
I'm the Covid 'lone star' ... 'Magic variants' & third waves are coming
Masks & social distancing, are staying, even if you've had a vaccine
That's right 'brother Anthony'.
The Puppet Show Reality
I'm Whitty
STAY SCARED
CONTROL THE PEOPLE
DESTROY LIVES

Figure 17: Artist Neil Hague's version of the 'Covid' hierarchy.

Human batteries

A state of such inversion does have its consequences, however. The level of disconnection from the Source of All means that you withdraw from that source of energetic sustenance and creativity. This means that you have to find your own supply of energetic power and it has – *us*. When the Morpheus character in the first *Matrix* movie held up a battery he spoke a profound truth when he said: 'The Matrix is a computer-generated dream world built to keep us under control in order to change the human being into one of

these.' The statement was true in all respects. We do live in a technologically-generated virtual reality simulation (more very shortly) and we have been manipulated to be an energy source for Archontic consciousness. The Disney-Pixar animated movie *Monsters, Inc*. in 2001 symbolised the dynamic when monsters in their world had no energy source and they would enter the human world to terrify children in their beds, catch the child's scream, terror (low-vibrational frequencies), and take that energy back to power the monster world. The lead character you might remember was a single giant eye and the symbolism of the Cult's all-seeing eye was obvious. Every thought and emotion is broadcast as a frequency unique to that thought and emotion. Feelings of love and joy, empathy and compassion, are high, quick, frequencies while fear, depression, anxiety, suffering and hate are low, slow, dense frequencies. Which kind do you think Archontic consciousness can connect with and absorb? In such a low and dense frequency state there's no way it can connect with the energy of love and joy. Archons can only feed off energy compatible with their own frequency and they and their Cult agents want to delete the human world of love and joy and manipulate the transmission of low vibrational frequencies through low-vibrational human mental and emotional states. *We are their energy source*. Wars are energetic banquets to the Archons – a world war even more so – and think how much low-frequency mental and emotional energy has been generated from the consequences for humanity of the 'Covid' hoax orchestrated by Archons incarnate like Gates.

The ancient practice of human sacrifice 'to the gods', continued in secret today by the Cult, is based on the same principle. 'The gods' are Archontic consciousness in different forms and the sacrifice is induced into a state of intense terror to generate the energy the Archontic frequency can absorb. Incarnate Archons in the ritual drink the blood which contains an adrenaline they crave which floods into the bloodstream when people are terrorised. Most of the sacrifices, ancient and modern, are children and the theme of 'sacrificing young virgins to the gods' is just code for children. They

have a particular pre-puberty energy that Archons want more than anything and the energy of the young in general is their target. The California Department of Education wants students to chant the names of Aztec gods (Archontic gods) once worshipped in human sacrifice rituals in a curriculum designed to encourage them to 'challenge racist, bigoted, discriminatory, imperialist/colonial beliefs', join 'social movements that struggle for social justice', and 'build new possibilities for a post-racist, post-systemic racism society'. It's the usual Woke crap that inverts racism and calls it anti-racism. In this case solidarity with 'indigenous tribes' is being used as an excuse to chant the names of 'gods' to which people were sacrificed (and still are in secret). What an example of Woke's inability to see beyond black and white, us and them, They condemn the colonisation of these tribal cultures by Europeans (quite right), but those cultures sacrificing people including children to their 'gods', and mass murdering untold numbers as the Aztecs did, is just fine. One chant is to the Aztec god Tezcatlipoca who had a man sacrificed to him in the 5th month of the Aztec calendar. His heart was cut out and he was eaten. Oh, that's okay then. Come on children … after three … Other sacrificial 'gods' for the young to chant their allegiance include Quetzalcoatl, Huitzilopochtli and Xipe Totec. The curriculum says that 'chants, affirmations, and energizers can be used to bring the class together, build unity around ethnic studies principles and values, and to reinvigorate the class following a lesson that may be emotionally taxing or even when student engagement may appear to be low'. Well, that's the cover story, anyway. Chanting and mantras are the repetition of a particular frequency generated from the vocal cords and chanting the names of these Archontic 'gods' tunes you into their frequency. That is the last thing you want when it allows for energetic synchronisation, attachment and perceptual influence. Initiates chant the names of their 'Gods' in their rituals for this very reason.

Vampires of the Woke

Paedophilia is another way that Archons absorb the energy of children. Paedophiles possessed by Archontic consciousness are used as the conduit during sexual abuse for discarnate Archons to vampire the energy of the young they desire so much. Stupendous numbers of children disappear every year never to be seen again although you would never know from the media. Imagine how much low-vibrational energy has been generated by children during the 'Covid' hoax when so many have become depressed and psychologically destroyed to the point of killing themselves. Shocking numbers of children are now taken by the state from loving parents to be handed to others. I can tell you from long experience of researching this since 1996 that many end up with paedophiles and assets of the Cult through corrupt and Cult-owned social services which in the reframing era has hired many psychopaths and emotionless automatons to do the job. Children are even stolen to order using spurious reasons to take them by the corrupt and secret (because they're corrupt) 'family courts'. I have written in detail in other books, starting with *The Biggest Secret* in 1997, about the ubiquitous connections between the political, corporate, government, intelligence and military elites (Cult operatives) and Satanism and paedophilia. If you go deep enough both networks have an interlocking leadership. The Woke mentality has been developed by the Cult for many reasons: To promote almost every aspect of its agenda; to hijack the traditional political left and turn it fascist; to divide and rule; and to target agenda pushbackers. But there are other reasons which relate to what I am describing here. How many happy and joyful Wokers do you ever see especially at the extreme end? They are a mental and psychological mess consumed by emotional stress and constantly emotionally cocked for the next explosion of indignation at someone referring to a female as a female. They are walking, talking, batteries as Morpheus might say emitting frequencies which both enslave them in low-vibrational bubbles of perceptual limitation and feed the Archons. Add to this the hatred claimed to be love; fascism claimed to 'anti-fascism', racism claimed to be 'anti-racism';

exclusion claimed to inclusion; and the abuse-filled Internet trolling. You have a purpose-built Archontic energy system with not a wind turbine in sight and all founded on Archontic *inversion*. We have whole generations now manipulated to serve the Archons with their actions and energy. They will be doing so their entire adult lives unless they snap out of their Archon-induced trance. Is it really a surprise that Cult billionaires and corporations put so much money their way? Where is the energy of joy and laughter, including laughing at yourself which is confirmation of your own emotional security? Mark Twain said: 'The human race has one really effective weapon, and that is laughter.' We must use it all the time. Woke has destroyed comedy because it has no humour, no joy, sense of irony, or self-deprecation. Its energy is dense and intense. *Mmmmm*, lunch says the Archontic frequency. Rudolf Steiner (1861-1925) was the Austrian philosopher and famous esoteric thinker who established Waldorf education or Steiner schools to treat children like unique expressions of consciousness and not minds to be programmed with the perceptions determined by authority. I'd been writing about this energy vampiring for decades when I was sent in 2016 a quote by Steiner. He was spot on:

> There are beings in the spiritual realms for whom anxiety and fear emanating from human beings offer welcome food. When humans have no anxiety and fear, then these creatures starve. If fear and anxiety radiates from people and they break out in panic, then these creatures find welcome nutrition and they become more and more powerful. These beings are hostile towards humanity. Everything that feeds on negative feelings, on anxiety, fear and superstition, despair or doubt, are in reality hostile forces in super-sensible worlds, launching cruel attacks on human beings, while they are being fed ... These are exactly the feelings that belong to contemporary culture and materialism; because it estranges people from the spiritual world, it is especially suited to evoke hopelessness and fear of the unknown in people, thereby calling up the above mentioned hostile forces against them.

Pause for a moment from this perspective and reflect on what has happened in the world since the start of 2020. Not only will pennies drop, but billion dollar bills. We see the same theme from Don Juan Matus, a Yaqui Indian shaman in Mexico and the information source for Peruvian-born writer, Carlos Castaneda, who wrote a series of

books from the 1960s to 1990s. Don Juan described the force manipulating human society and his name for the Archons was the predator:

> We have a predator that came from the depths of the cosmos and took over the rule of our lives. Human beings are its prisoners. The predator is our lord and master. It has rendered us docile, helpless. If we want to protest, it suppresses our protest. If we want to act independently, it demands that we don't do so ... indeed we are held prisoner!
>
> They took us over because we are food to them, and they squeeze us mercilessly because we are their sustenance. Just as we rear chickens in coops, the predators rear us in human coops, humaneros. Therefore, their food is always available to them.
>
> Different cultures, different eras, same recurring theme.

The 'ennoia' dilemma

Nag Hammadi Gnostic manuscripts say that Archon consciousness has no 'ennoia'. This is directly translated as 'intentionality', but I'll use the term 'creative imagination'. The *All That Is* in awareness of itself is the source of all creativity – all possibility – and the more disconnected you are from that source the more you are subsequently denied 'creative imagination'. Given that Archon consciousness is almost entirely disconnected it severely lacks creativity and has to rely on far more mechanical processes of thought and exploit the creative potential of those that do have 'ennoia'. You can see cases of this throughout human society. Archon consciousness almost entirely dominates the global banking system and if we study how that system works you will appreciate what I mean. Banks manifest 'money' out of nothing by issuing lines of 'credit' which is 'money' that has never, does not, and will never exist except in theory. It's a confidence trick. If you think 'credit' figures-on-a-screen 'money' is worth anything you accept it as payment. If you don't then the whole system collapses through lack of confidence in the value of that 'money'. Archontic bankers with no 'ennoia' are 'lending' 'money' that doesn't exist to humans that *do* have creativity – those that have the inspired ideas and create businesses and products. Archon banking feeds off human creativity

which it controls through 'money' creation and debt. Humans have the creativity and Archons exploit that for their own benefit and control while having none themselves. Archon Internet platforms like Facebook claim joint copyright of everything that creative users post and while Archontic minds like Zuckerberg may officially head that company it will be human creatives on the staff that provide the creative inspiration. When you have limitless 'money' you can then buy other companies established by creative humans. Witness the acquisition record of Facebook, Google and their like. Survey the Archon-controlled music industry and you see non-creative dark suit executives making their fortune from the human creativity of their artists. The cases are endless. Research the history of people like Gates and Zuckerberg and how their empires were built on exploiting the creativity of others. Archon minds cannot create out of nothing, but they are skilled (because they have to be) in what Gnostic texts call 'countermimicry'. They can imitate, but not innovate. Sabbatians trawl the creativity of others through backdoors they install in computer systems through their cybersecurity systems. Archon-controlled China is globally infamous for stealing intellectual property and I remember how Hong Kong, now part of China, became notorious for making counterfeit copies of the creativity of others – 'countermimicry'. With the now pervasive and all-seeing surveillance systems able to infiltrate any computer you can appreciate the potential for Archons to vampire the creativity of humans. Author John Lamb Lash wrote in his book about the Nag Hammadi texts, *Not In His Image*:

> Although they cannot originate anything, because they lack the divine factor of ennoia (intentionality), Archons can imitate with a vengeance. Their expertise is simulation (HAL, virtual reality). The Demiurge [Yaldabaoth] fashions a heaven world copied from the fractal patterns [of the original] ... His construction is celestial kitsch, like the fake Italianate villa of a Mafia don complete with militant angels to guard every portal.

This brings us to something that I have been speaking about since the turn of the millennium. Our reality is a simulation; a virtual reality that we think is real. No, I'm not kidding.

Human reality? Well, virtually

I had pondered for years about whether our reality is 'real' or some kind of construct. I remembered being immensely affected on a visit as a small child in the late 1950s to the then newly-opened Planetarium on the Marylebone Road in London which is now closed and part of the adjacent Madame Tussauds wax museum. It was in the middle of the day, but when the lights went out there was the night sky projected in the Planetarium's domed ceiling and it appeared to be so real. The experience never left me and I didn't know why until around the turn of the millennium when I became certain that our 'night sky' and entire reality is a projection, a virtual reality, akin to the illusory world portrayed in the *Matrix* movies. I looked at the sky one day in this period and it appeared to me like the domed roof of the Planetarium. The release of the first *Matrix* movie in 1999 also provided a synchronistic and perfect visual representation of where my mind had been going for a long time. I hadn't come across the Gnostic Nag Hammadi texts then. When I did years later the correlation was once again astounding. As I read Gnostic accounts from 1,600 years and more earlier it was clear that they were describing the same simulation phenomenon. They tell how the Yaldabaoth 'Demiurge' and Archons created a 'bad copy' of original reality to rule over all that were captured by its illusions and the body was a prison to trap consciousness in the 'bad copy' fake reality. Read how Gnostics describe the 'bad copy' and update that to current times and they are referring to what we would call today a virtual reality simulation.

Author John Lamb Lash said 'the Demiurge fashions a heaven world copied from the fractal patterns' of the original through expertise in 'HAL' or virtual reality simulation. Fractal patterns are part of the energetic information construct of our reality, a sort of blueprint. If these patterns were copied in computer terms it would indeed give you a copy of a 'natural' reality in a non-natural frequency and digital form. The principle is the same as making a copy of a website. The original website still exists, but now you can change the copy version to make it whatever you like and it can

become very different to the original website. Archons have done this with our reality, a *synthetic* copy of prime reality that still exists beyond the frequency walls of the simulation. Trapped within the illusions of this synthetic Matrix, however, were and are human consciousness and other expressions of prime reality and this is why the Archons via the Cult are seeking to make the human body synthetic and give us synthetic AI minds to complete the job of turning the entire reality synthetic including what we perceive to be the natural world. To quote Kurzweil: 'Nanobots will infuse all the matter around us with information. Rocks, trees, everything will become these intelligent creatures.' Yes, *synthetic* 'creatures' just as 'Covid' and other genetically-manipulating 'vaccines' are designed to make the human body synthetic. From this perspective it is obvious why Archons and their Cult are so desperate to infuse synthetic material into every human with their 'Covid' scam.

Let there be (electromagnetic) light

Yaldabaoth, the force that created the simulation, or Matrix, makes sense of the Gnostic reference to 'The Great Architect' and its use by Cult Freemasonry as the name of its deity. The designer of the Matrix in the movies is called 'The Architect' and that trilogy is jam-packed with symbolism relating to these subjects. I have contended for years that the angry Old Testament God (Yaldabaoth) is the 'God' being symbolically 'quoted' in the opening of Genesis as 'creating the world'. This is not the creation of prime reality – it's the creation of the *simulation*. The Genesis 'God' says: 'Let there be Light: and there was light.' But what is this 'Light'? I have said for decades that the speed of light (186,000 miles per second) is not the fastest speed possible as claimed by mainstream science and is in fact the frequency walls or outer limits of the Matrix. You can't have a fastest or slowest anything within all possibility when everything is possible. The human body is encoded to operate within the speed of light or *within the simulation* and thus we see only the tiny frequency band of visible *light*. Near-death experiencers who perceive reality outside the body during temporary 'death' describe a very different

form of light and this is supported by the Nag Hammadi texts. Prime reality beyond the simulation ('Upper Aeons' to the Gnostics) is described as a realm of incredible beauty, bliss, love and harmony – a realm of 'watery light' that is so powerful 'there are no shadows'. Our false reality of Archon control, which Gnostics call the 'Lower Aeons', is depicted as a realm with a different kind of 'light' and described in terms of chaos, 'Hell', 'the Abyss' and 'Outer Darkness', where trapped souls are tormented and manipulated by demons (relate that to the 'Covid' hoax alone). The watery light theme can be found in near-death accounts and it is not the same as *simulation* 'light' which is electromagnetic or radiation light within the speed of light – the 'Lower Aeons'. Simulation 'light' is the 'luminous fire' associated by Gnostics with the Archons. The Bible refers to Yaldabaoth as 'that old serpent, called the Devil, and Satan, which deceiveth the whole world' (Revelation 12:9). I think that making a simulated copy of prime reality ('countermimicry') and changing it dramatically while all the time manipulating humanity to believe it to be real could probably meet the criteria of deceiving the whole world. Then we come to the Cult god Lucifer – the *Light Bringer*. Lucifer is symbolic of Yaldabaoth, the bringer of radiation light that forms the bad copy simulation within the speed of light. 'He' is symbolised by the lighted torch held by the Statue of Liberty and in the name 'Illuminati'. Sabbatian-Frankism declares that Lucifer is the true god and Lucifer is the real god of Freemasonry honoured as their 'Great or Grand Architect of the Universe' (simulation).

I would emphasise, too, the way Archontic technologically-generated luminous fire of radiation has deluged our environment since I was a kid in the 1950s and changed the nature of The Field with which we constantly interact. Through that interaction technological radiation is changing us. The Smart Grid is designed to operate with immense levels of communication power with 5G expanding across the world and 6G, 7G, in the process of development. Radiation is the simulation and the Archontic manipulation system. Why wouldn't the Archon Cult wish to unleash radiation upon us to an ever-greater extreme to form

Kurzweil's 'cloud'? The plan for a synthetic human is related to the need to cope with levels of radiation beyond even anything we've seen so far. Biological humans would not survive the scale of radiation they have in their script. The Smart Grid is a technological sub-reality within the technological simulation to further disconnect five-sense perception from expanded consciousness. It's a technological prison of the mind.

Infusing the 'spirit of darkness'

A recurring theme in religion and native cultures is the manipulation of human genetics by a non-human force and most famously recorded as the biblical 'sons of god' (the god*s* plural in the original) who interbred with the daughters of men. The Nag Hammadi *Apocryphon of John* tells the same story this way:

> He [Yaldabaoth] sent his angels [Archons/demons] to the daughters of men, that they might take some of them for themselves and raise offspring for their enjoyment. And at first they did not succeed. When they had no success, they gathered together again and they made a plan together ... And the angels changed themselves in their likeness into the likeness of their mates, filling them with the spirit of darkness, which they had mixed for them, and with evil ... And they took women and begot children out of the darkness according to the likeness of their spirit.

Possession when a discarnate entity takes over a human body is an age-old theme and continues today. It's very real and I've seen it. Satanic and secret society rituals can create an energetic environment in which entities can attach to initiates and I've heard many stories of how people have changed their personality after being initiated even into lower levels of the Freemasons. I have been inside three Freemasonic temples, one at a public open day and two by just walking in when there was no one around to stop me. They were in Ryde, the town where I live, Birmingham, England, when I was with a group, and Boston, Massachusetts. They all felt the same energetically – dark, dense, low-vibrational and sinister. Demonic attachment can happen while the initiate has no idea what is going on. To them it's just a ritual to get in the Masons and do a bit of good

business. In the far more extreme rituals of Satanism human possession is even more powerful and they are designed to make possession possible. The hierarchy of the Cult is dictated by the power and perceived status of the possessing Archon. In this way the Archon hierarchy becomes the Cult hierarchy. Once the entity has attached it can influence perception and behaviour and if it attaches to the extreme then so much of its energy (information) infuses into the body information field that the hologram starts to reflect the nature of the possessing entity. This is the *Exorcist* movie type of possession when facial features change and it's known as shapeshifting. Islam's Jinn are said to be invisible tricksters who change shape, 'whisper', confuse and take human form. These are all traits of the Archons and other versions of the same phenomenon. Extreme possession could certainty infuse the 'spirit of darkness' into a partner during sex as the Nag Hammadi texts appear to describe. Such an infusion can change genetics which is also energetic information. Human genetics is information and the 'spirit of darkness' is information. Mix one with the other and change must happen. Islam has the concept of a 'Jinn baby' through possession of the mother and by Jinn taking human form. There are many ways that human genetics can be changed and remember that Archons have been aware all along of advanced techniques to do this. What is being done in human society today – and far more – was known about by Archons at the time of the 'fallen ones' and their other versions described in religions and cultures.

Archons and their human-world Cult are obsessed with genetics as we see today and they know this dictates how information is processed into perceived reality during a human life. They needed to produce a human form that would decode the simulation and this is symbolically known as 'Adam and Eve' who left the 'garden' (prime reality) and 'fell' into Matrix reality. The simulation is not a 'physical' construct (there is no 'physical'); it is a source of information. Think Wi-Fi again. The simulation is an energetic field encoded with information and body-brain systems are designed to decode that information encoded in wave or frequency form which

is transmitted to the brain as electrical signals. These are decoded by the brain to construct our sense of reality – an illusory 'physical' world that only exists in the brain or the mind. Virtual reality games mimic this process using the same sensory decoding system. Information is fed to the senses to decode a virtual reality that can appear so real, but isn't (Figs 18 and 19). Some scientists believe – and I agree with them – that what we perceive as 'physical' reality only exists when we are looking or observing. The act of perception or focus triggers the decoding systems which turn waveform information into holographic reality. When we are not observing something our reality reverts from a holographic state to a waveform state. This relates to the same principle as a falling tree not making a noise unless someone is there to hear it or decode it. The concept makes sense from the simulation perspective. A computer is not decoding all the information in a Wi-Fi field all the time and only decodes or brings into reality on the screen that part of Wi-Fi that it's decoding – focusing upon – at that moment.

Figure 18: Virtual reality technology 'hacks' into the body's five-sense decoding system.

Figure 19: The result can be experienced as very 'real'.

Interestingly, Professor Donald Hoffman at the Department of Cognitive Sciences at the University of California, Irvine, says that our experienced reality is like a computer interface that shows us only the level with which we interact while hiding all that exists beyond it: 'Evolution shaped us with a user interface that hides the truth. Nothing that we see is the truth – the very language of space and time and objects is the wrong language to describe reality.' He is correct in what he says on so many levels. Space and time are not a universal reality. They are a phenomenon of decoded *simulation* reality as part of the process of enslaving our sense of reality. Near-death experiencers report again and again how space and time did not exist as we perceive them once they were free of the body – body decoding systems. You can appreciate from this why Archons and their Cult are so desperate to entrap human attention in the five senses where we are in the Matrix and of the Matrix. Opening your mind to expanded states of awareness takes you beyond the information confines of the simulation and you become aware of knowledge and insights denied to you before. This is what we call 'awakening' – *awakening from the Matrix* – and in the final chapter I will relate this to current events.

Where are the 'aliens'?

A simulation would explain the so-called 'Fermi Paradox' named after Italian physicist Enrico Fermi (1901-1954) who created the first nuclear reactor. He considered the question of why there is such a lack of extraterrestrial activity when there are so many stars and planets in an apparently vast universe; but what if the night sky that we see, or think we do, is a simulated projection as I say? If you control the simulation and your aim is to hold humanity fast in essential ignorance would you want other forms of life including advanced life coming and going sharing information with humanity? Or would you want them to believe they were isolated and apparently alone? Themes of human isolation and apartness are common whether they be the perception of a lifeless universe or the fascist isolation laws of the 'Covid' era. Paradoxically the very

existence of a simulation means that we are not alone when some force had to construct it. My view is that experiences that people have reported all over the world for centuries with Reptilians and Grey entities are Archon phenomena as Nag Hammadi texts describe; and that benevolent 'alien' interactions are non-human groups that come in and out of the simulation by overcoming Archon attempts to keep them out. It should be highlighted, too, that Reptilians and Greys are obsessed with *genetics* and *technology* as related by cultural accounts and those who say they have been abducted by them. Technology is their way of overcoming some of the limitations in their creative potential and our technology-driven and controlled human society of today is *arch*etypical Archon-Reptilian-Grey modus operandi. Technocracy is really *Archon*tocracy. The Universe does not have to be as big as it appears with a simulation. There is no space or distance only information decoded into holographic reality. What we call 'space' is only the absence of holographic 'objects' and that 'space' is The Field of energetic information which connects everything into a single whole. The same applies with the artificially-generated information field of the simulation. The Universe is not big or small as a physical reality. It is decoded information, that's all, and its perceived size is decided by the way the simulation is encoded to make it appear. The entire night sky as we perceive it only exists in our brain and so where are those 'millions of light years'? The 'stars' on the ceiling of the Planetarium looked a vast distance away.

There's another point to mention about 'aliens'. I have been highlighting since the 1990s the plan to stage a fake 'alien invasion' to justify the centralisation of global power and a world military. Nazi scientist Werner von Braun, who was taken to America by Operation Paperclip after World War Two to help found NASA, told his American assistant Dr Carol Rosin about the Cult agenda when he knew he was dying in 1977. Rosin said that he told her about a sequence that would lead to total human control by a one-world government. This included threats from terrorism, rogue nations, meteors and asteroids before finally an 'alien invasion'. All of these

things, von Braun said, would be bogus and what I would refer to as a No-Problem-Reaction-Solution. Keep this in mind when 'the aliens are coming' is the new mantra. The aliens are not coming – they are *already here* and they have infiltrated human society while looking human. French-Canadian investigative journalist Serge Monast said in 1994 that he had uncovered a NASA/military operation called Project Blue Beam which fits with what Werner von Braun predicted. Monast died of a 'heart attack' in 1996 the day after he was arrested and spent a night in prison. He was 51. He said Blue Beam was a plan to stage an alien invasion that would include religious figures beamed holographically into the sky as part of a global manipulation to usher in a 'new age' of worshipping what I would say is the Cult 'god' Yaldabaoth in a one-world religion. Fake holographic asteroids are also said to be part of the plan which again syncs with von Braun. How could you stage an illusory threat from asteroids unless they were holographic inserts? This is pretty straightforward given the advanced technology outside the public arena and the fact that our 'physical' reality is holographic anyway. Information fields would be projected and we would decode them into the illusion of a 'physical' asteroid. If they can sell a global 'pandemic' with a 'virus' that doesn't exist what will humans not believe if government and media tell them?

All this is particularly relevant as I write with the Pentagon planning to release in June, 2021, information about 'UFO sightings'. I have been following the UFO story since the early 1990s and the common theme throughout has been government and military denials and cover up. More recently, however, the Pentagon has suddenly become more talkative and apparently open with Air Force pilot radar images released of unexplained craft moving and changing direction at speeds well beyond anything believed possible with human technology. Then, in March, 2021, former Director of National Intelligence John Ratcliffe said a Pentagon report months later in June would reveal a great deal of information about UFO sightings unknown to the public. He said the report would have 'massive implications'. The order to do this was included bizarrely

in a $2.3 trillion 'coronavirus' relief and government funding bill passed by the Trump administration at the end of 2020. I would add some serious notes of caution here. I have been pointing out since the 1990s that the US military and intelligence networks have long had craft – 'flying saucers' or anti-gravity craft – which any observer would take to be extraterrestrial in origin. Keeping this knowledge from the public allows craft flown by *humans* to be perceived as alien visitations. I am not saying that 'aliens' do not exist. I would be the last one to say that, but we have to be streetwise here. President Ronald Reagan told the UN General Assembly in 1987: 'I occasionally think how quickly our differences worldwide would vanish if we were facing an alien threat from outside this world.' That's the idea. Unite against a common 'enemy' with a common purpose behind your 'saviour force' (the Cult) as this age-old technique of mass manipulation goes global.

Science moves this way …

I could find only one other person who was discussing the simulation hypothesis publicly when I concluded it was real. This was Nick Bostrom, a Swedish-born philosopher at the University of Oxford, who has explored for many years the possibility that human reality is a computer simulation although his version and mine are not the same. Today the simulation and holographic reality hypothesis have increasingly entered the scientific mainstream. Well, the more open-minded mainstream, that is. Here are a few of the ever-gathering examples. American nuclear physicist Silas Beane led a team of physicists at the University of Bonn in Germany pursuing the question of whether we live in a simulation. They concluded that we probably do and it was likely based on a lattice of cubes. They found that cosmic rays align with that specific pattern. The team highlighted the Greisen–Zatsepin–Kuzmin (GZK) limit which refers to cosmic ray particle interaction with cosmic background radiation that creates an apparent boundary for cosmic ray particles. They say in a paper entitled 'Constraints on the Universe as a Numerical Simulation' that this 'pattern of constraint' is exactly what you

would find with a computer simulation. They also made the point that a simulation would create its own 'laws of physics' that would limit possibility. I've been making the same point for decades that the *perceived* laws of physics relate only to this reality, or what I would later call the simulation. When designers write codes to create computer and virtual reality games they are the equivalent of the laws of physics for that game. Players interact within the limitations laid out by the coding. In the same way those who wrote the codes for the simulation decided the laws of physics that would apply. These can be overridden by expanded states of consciousness, but not by those enslaved in only five-sense awareness where simulation codes rule. Overriding the codes is what people call 'miracles'. They are not. They are bypassing the encoded limits of the simulation. A population caught in simulation perception would have no idea that this was their plight. As the Bonn paper said: 'Like a prisoner in a pitch-black cell we would not be able to see the "walls" of our prison,' That's true if people remain mesmerised by the five senses. Open to expanded awareness and those walls become very clear. The main one is the speed of light.

American theoretical physicist James Gates is another who has explored the simulation question and found considerable evidence to support the idea. Gates was Professor of Physics at the University of Maryland, Director of The Center for String and Particle Theory, and on Barack Obama's Council of Advisors on Science and Technology. He and his team found *computer codes* of digital data embedded in the fabric of our reality. They relate to on-off electrical charges of 1 and 0 in the binary system used by computers. 'We have no idea what they are doing there', Gates said. They found within the energetic fabric mathematical sequences known as error-correcting codes or block codes that 'reboot' data to its original state or 'default settings' when something knocks it out of sync. Gates was asked if he had found a set of equations embedded in our reality indistinguishable from those that drive search engines and browsers and he said: 'That is correct.' Rich Terrile, director of the Centre for Evolutionary Computation and Automated Design at NASA's Jet

Propulsion Laboratory, has said publicly that he believes the Universe is a digital hologram that must have been created by a form of intelligence. I agree with that in every way. Waveform information is delivered electrically by the senses to the brain which constructs a *digital* holographic reality that we call the 'world'. This digital level of reality can be read by the esoteric art of numerology. Digital holograms are at the cutting edge of holographics today. We have digital technology everywhere designed to access and manipulate our digital level of perceived reality. Synthetic mRNA in 'Covid vaccines' has a digital component to manipulate the body's digital 'operating system'.

Reality is numbers

How many know that our reality can be broken down to numbers and codes that are the same as computer games? Max Tegmark, a physicist at the Massachusetts Institute of Technology (MIT), is the author of *Our Mathematical Universe* in which he lays out how reality can be entirely described by numbers and maths in the way that a video game is encoded with the 'physics' of computer games. Our world and computer virtual reality are essentially the same. Tegmark imagines the perceptions of characters in an advanced computer game when the graphics are so good they don't know they are in a game. They think they can bump into real objects (electromagnetic resistance in our reality), fall in love and feel emotions like excitement. When they began to study the apparently 'physical world' of the video game they would realise that everything was made of pixels (which have been found in our energetic reality as must be the case when on one level our world is digital). What computer game characters thought was physical 'stuff', Tegmark said, could actually be broken down into numbers:

> And we're exactly in this situation in our world. We look around and it doesn't seem that mathematical at all, but everything we see is made out of elementary particles like quarks and electrons. And what properties does an electron have? Does it have a smell or a colour or a texture? No! ... We physicists have come up with geeky names for [Electron] properties, like

electric charge, or spin, or lepton number, but the electron doesn't care what we call it, the properties are just numbers.

This is the illusory reality Gnostics were describing. This is the simulation. The A, C, G, and T codes of DNA have a binary value – A and C = 0 while G and T = 1. This has to be when the simulation is digital and the body must be digital to interact with it. Recurring mathematical sequences are encoded throughout reality and the body. They include the Fibonacci sequence in which the two previous numbers are added to get the next one, as in ... 1, 1, 2, 3, 5, 8, 13, 21, 34, 55, etc. The sequence is encoded in the human face and body, proportions of animals, DNA, seed heads, pine cones, trees, shells, spiral galaxies, hurricanes and the number of petals in a flower. The list goes on and on. There are fractal patterns – a 'never-ending pattern that is infinitely complex and self-similar across all scales in the as above, so below, principle of holograms. These and other famous recurring geometrical and mathematical sequences such as Phi, Pi, Golden Mean, Golden Ratio and Golden Section are *computer codes* of the simulation. I had to laugh and give my head a shake the day I finished this book and it went into the production stage. I was sent an article in *Scientific American* published in April, 2021, with the headline 'Confirmed! We Live in a Simulation'. Two decades after I first said our reality is a simulation and the speed of light is it's outer limit the article suggested that we do live in a simulation and that the speed of light is its outer limit. I left school at 15 and never passed a major exam in my life while the writer was up to his eyes in qualifications. As I will explain in the final chapter *knowing* is far better than thinking and they come from very different sources. The article rightly connected the speed of light to the processing speed of the 'Matrix' and said what has been in my books all this time … 'If we are in a simulation, as it appears, then space is an abstract property written in code. It is not real'. No it's not and if we live in a simulation something created it and it wasn't *us*. 'That David Icke says we are manipulated by aliens' – he's crackers.'

Wow …

The reality that humanity thinks is so real is an illusion. Politicians, governments, scientists, doctors, academics, law enforcement, media, school and university curriculums, on and on, are all founded on a world that *does not exist* except as a simulated prison cell. Is it such a stretch to accept that 'Covid' doesn't exist when our entire 'physical' reality doesn't exist? Revealed here is the knowledge kept under raps in the Cult networks of compartmentalised secrecy to control humanity's sense of reality by inducing the population to believe in a reality that's not real. If it wasn't so tragic in its experiential consequences the whole thing would be hysterically funny. None of this is new to Renegade Minds. Ancient Greek philosopher Plato (about 428 to about 347BC) was a major influence on Gnostic belief and he described the human plight thousands of years ago with his Allegory of the Cave. He told the symbolic story of prisoners living in a cave who had never been outside. They were chained and could only see one wall of the cave while behind them was a fire that they could not see. Figures walked past the fire casting shadows on the prisoners' wall and those moving shadows became their sense of reality. Some prisoners began to study the shadows and were considered experts on them (today's academics and scientists), but what they studied was only an illusion (today's academics and scientists). A prisoner escaped from the cave and saw reality as it really is. When he returned to report this revelation they didn't believe him, called him mad and threatened to kill him if he tried to set them free. Plato's tale is not only a brilliant analogy of the human plight and our illusory reality. It describes, too, the dynamics of the 'Covid' hoax. I have only skimmed the surface of these subjects here. The aim of this book is to crisply connect all essential dots to put what is happening today into its true context. All subject areas and their connections in this chapter are covered in great evidential detail in *Everything You Need To Know, But Have Never Been Told* and *The Answer*.

They say that bewildered people 'can't see the forest for the trees'. Humanity, however, can't see the forest for the *twigs*. The five senses

see only twigs while Renegade Minds can see the forest and it's the forest where the answers lie with the connections that reveals. Breaking free of perceptual programming so the forest can be seen is the way we turn all this around. Not breaking free is how humanity got into this mess. The situation may seem hopeless, but I promise you it's not. We are a perceptual heartbeat from paradise if only we knew.

CHAPTER TWELVE

Escaping Wetiko

Life is simply a vacation from the infinite
Dean Cavanagh

Renegade Minds weave the web of life and events and see common themes in the apparently random. They are always there if you look for them and their pursuit is aided by incredible synchronicity that comes when your mind is open rather than mesmerised by what it thinks it can see.

Infinite awareness is infinite possibility and the more of infinite possibility that we access the more becomes infinitely possible. That may be stating the apparently obvious, but it is a devastatingly-powerful fact that can set us free. We are a point of attention within an infinity of consciousness. The question is how much of that infinity do we choose to access? How much knowledge, insight, awareness, wisdom, do we want to connect with and explore? If your focus is only in the five senses you will be influenced by a fraction of infinite awareness. I mean a range so tiny that it gives new meaning to infinitesimal. Limitation of self-identity and a sense of the possible limit accordingly your range of consciousness. We are what we think we are. Life is what we think it is. The dream is the dreamer and the dreamer is the dream. Buddhist philosophy puts it this way: 'As a thing is viewed, so it appears.' Most humans live in the realm of touch, taste, see, hear, and smell and that's the limit of their sense of the possible and sense of self. Many will follow a religion and speak of a God in his heaven, but their lives are still

dominated by the five senses in their perceptions and actions. The five senses become the arbiter of everything. When that happens all except a smear of infinity is sealed away from influence by the rigid, unyielding, reality bubbles that are the five-sense human or Phantom Self. Archon Cult methodology is to isolate consciousness within five-sense reality – the simulation – and then program that consciousness with a sense of self and the world through a deluge of life-long information designed to instil the desired perception that allows global control. Efforts to do this have increased dramatically with identity politics as identity bubbles are squeezed into the minutiae of five-sense detail which disconnect people even more profoundly from the infinite 'I'.

Five-sense focus and self-identity are like a firewall that limits access to the infinite realms. You only perceive one radio or television station and no other. We'll take that literally for a moment. Imagine a vast array of stations giving different information and angles on reality, but you only ever listen to one. Here we have the human plight in which the population is overwhelmingly confined to CultFM. This relates only to the frequency range of CultFM and limits perception and insight to that band – limits *possibility* to that band. It means you are connecting with an almost imperceptibly minuscule range of possibility and creative potential within the infinite Field. It's a world where everything seems apart from everything else and where synchronicity is rare. Synchronicity is defined in the dictionary as 'the happening by chance of two or more related or similar events at the same time'. Use of 'by chance' betrays a complete misunderstanding of reality. Synchronicity is not 'by chance'. As people open their minds, or 'awaken' to use the term, they notice more and more coincidences in their lives, bits of 'luck', apparently miraculous happenings that put them in the right place at the right time with the right people. Days become peppered with 'fancy meeting you here' and 'what are the chances of that?' My entire life has been lived like this and ever more so since my own colossal awakening in 1990 and 91 which transformed my sense of reality. Synchronicity is not 'by chance'; it is by accessing expanded

realms of possibility which allow expanded potential for manifestation. People broadcasting the same vibe from the same openness of mind tend to be drawn 'by chance' to each other through what I call frequency magnetism and it's not only people. In the last more than 30 years incredible synchronicity has also led me through the Cult maze to information in so many forms and to crucial personal experiences. These 'coincidences' have allowed me to put the puzzle pieces together across an enormous array of subjects and situations. Those who have breached the bubble of five-sense reality will know exactly what I mean and this escape from the perceptual prison cell is open to everyone whenever they make that choice. This may appear super-human when compared with the limitations of 'human', but it's really our natural state. 'Human' as currently experienced is consciousness in an unnatural state of induced separation from the infinity of the whole. I'll come to how this transformation into unity can be made when I have described in more detail the force that holds humanity in servitude by denying this access to infinite self.

The Wetiko factor

I have been talking and writing for decades about the way five-sense mind is systematically barricaded from expanded awareness. I have used the analogy of a computer (five-sense mind) and someone at the keyboard (expanded awareness). Interaction between the computer and the operator is symbolic of the interaction between five-sense mind and expanded awareness. The computer directly experiences the Internet and the operator experiences the Internet via the computer which is how it's supposed to be – the two working as one. Archons seek to control that point where the operator connects with the computer to stop that interaction (Fig 20). Now the operator is banging the keyboard and clicking the mouse, but the computer is not responding and this happens when the computer is taken over – *possessed* – by an appropriately-named computer 'virus'. The operator has lost all influence over the computer which goes its own way making decisions under the control of the 'virus'. I have

just described the dynamic through which the force known to Gnostics as Yaldabaoth and Archons disconnects five-sense mind from expanded awareness to imprison humanity in perceptual servitude.

Figure 20: The mind 'virus' I have been writing about for decades seeks to isolate five-sense mind (the computer) from the true 'I'. (Image by Neil Hague).

About a year ago I came across a Native American concept of Wetiko which describes precisely the same phenomenon. Wetiko is the spelling used by the Cree and there are other versions including wintiko and windigo used by other tribal groups. They spell the name with lower case, but I see Wetiko as a proper noun as with Archons and prefer a capital. I first saw an article about Wetiko by writer and researcher Paul Levy which so synced with what I had been writing about the computer/operator disconnection and later the Archons. I then read his book, the fascinating *Dispelling Wetiko, Breaking the Spell of Evil*. The parallels between what I had concluded long before and the Native American concept of Wetiko were so clear and obvious that it was almost funny. For Wetiko see the Gnostic Archons for sure and the Jinn, the Predators, and every other name for a force of evil, inversion and chaos. Wetiko is the Native American name for the force that divides the computer from

the operator (Fig 21). Indigenous author Jack D. Forbes, a founder of the Native American movement in the 1960s, wrote another book about Wetiko entitled *Columbus And Other Cannibals – The Wetiko Disease of Exploitation, Imperialism, and Terrorism* which I also read. Forbes says that Wetiko refers to an evil person or spirit 'who terrorizes other creatures by means of terrible acts, including cannibalism'. Zulu shaman Credo Mutwa told me that African accounts tell how cannibalism was brought into the world by the Chitauri 'gods' – another manifestation of Wetiko. The distinction between 'evil person or spirit' relates to Archons/Wetiko possessing a human or acting as pure consciousness. Wetiko is said to be a sickness of the soul or spirit and a state of being that takes but gives nothing back – the Cult and its operatives perfectly described. Black Hawk, a Native American war leader defending their lands from confiscation, said European invaders had 'poisoned hearts' – Wetiko hearts – and that this would spread to native societies. Mention of the heart is very significant as we shall shortly see. Forbes writes: 'Tragically, the history of the world for the past 2,000 years is, in great part, the story of the epidemiology of the wetiko disease.' Yes, and much longer. Forbes is correct when he says: 'The wetikos destroyed Egypt and Babylon and Athens and Rome and Tenochtitlan [capital of the Aztec empire] and perhaps now they will destroy the entire earth.' Evil, he said, is the number one export of a Wetiko culture – see its globalisation with 'Covid'. Constant war, mass murder, suffering of all kinds, child abuse, Satanism, torture and human sacrifice are all expressions of Wetiko and the Wetiko possessed. The world is Wetiko made manifest, *but it doesn't have to be*. There is a way out of this even now.

Figure 21: The mind 'virus' is known to Native Americans as 'Wetiko'. (Image by Neil Hague).

Cult of Wetiko

Wetiko is the Yaldabaoth frequency distortion that seeks to attach to human consciousness and absorb it into its own. Once this connection is made Wetiko can drive the perceptions of the target which they believe to be coming from their own mind. All the horrors of history and today from mass killers to Satanists, paedophiles like Jeffrey Epstein and other psychopaths, are the embodiment of Wetiko and express its state of being in all its grotesqueness. The Cult is Wetiko incarnate, Yaldabaoth incarnate, and it seeks to facilitate Wetiko assimilation of humanity in totality into its distortion by manipulating the population into low frequency states that match its own. Paul Levy writes: 'Holographically enforced within the psyche of every human being the wetiko virus pervades and underlies the entire field of consciousness, and can therefore potentially manifest through any one of us at any moment if we are not mindful.' The 'Covid' hoax has achieved this with many people, but others have not fallen into Wetiko's frequency lair. Players in the 'Covid' human catastrophe including Gates, Schwab, Tedros, Fauci, Whitty, Vallance, Johnson, Hancock, Ferguson, Drosten, and all the rest, including the psychopath psychologists, are expressions of Wetiko. This is why

they have no compassion or empathy and no emotional consequence for what they do that would make them stop doing it. Observe all the people who support the psychopaths in authority against the Pushbackers despite the damaging impact the psychopaths have on their own lives and their family's lives. You are again looking at Wetiko possession which prevents them seeing through the lies to the obvious scam going on. *Why can't they see it?* Wetiko won't let them see it. The perceptual divide that has now become a chasm is between the Wetikoed and the non-Wetikoed.

Paul Levy describes Wetiko in the same way that I have long described the Archontic force. They are the same distorted consciousness operating across dimensions of reality: '… the subtle body of wetiko is not located in the third dimension of space and time, literally existing in another dimension … it is able to affect ordinary lives by mysteriously interpenetrating into our three-dimensional world.' Wetiko does this through its incarnate representatives in the Cult and by weaving itself into The Field which on our level of reality is the electromagnetic information field of the simulation or Matrix. More than that, the simulation *is* Wetiko / Yaldabaoth. Caleb Scharf, Director of Astrobiology at Columbia University, has speculated that 'alien life' could be so advanced that it has transcribed itself into the quantum realm to become what we call physics. He said intelligence indistinguishable from the fabric of the Universe would solve many of its greatest mysteries:

> Perhaps hyper-advanced life isn't just external. Perhaps it's already all around. It is embedded in what we perceive to be physics itself, from the root behaviour of particles and fields to the phenomena of complexity and emergence ... In other words, life might not just be in the equations. It might BE the equations [My emphasis].

Scharf said it is possible that 'we don't recognise advanced life because it forms an integral and unsuspicious part of what we've considered to be the natural world'. I agree. Wetiko/Yaldabaoth *is* the simulation. We are literally in the body of the beast. But that doesn't mean it has to control us. We all have the power to overcome Wetiko

influence and the Cult knows that. I doubt it sleeps too well because it knows that.

Which Field?

This, I suggest, is how it all works. There are two Fields. One is the fierce electromagnetic light of the Matrix within the speed of light; the other is the 'watery light' of The Field beyond the walls of the Matrix that connects with the Great Infinity. Five-sense mind and the decoding systems of the body attach us to the Field of Matrix light. They have to or we could not experience this reality. Five-sense mind sees only the Matrix Field of information while our expanded consciousness is part of the Infinity Field. When we open our minds, and most importantly our hearts, to the Infinity Field we have a mission control which gives us an expanded perspective, a road map, to understand the nature of the five-sense world. If we are isolated only in five-sense mind there is no mission control. We're on our own trying to understand a world that's constantly feeding us information to ensure we do not understand. People in this state can feel 'lost' and bewildered with no direction or radar. You can see ever more clearly those who are influenced by the Fields of Big Infinity or little five-sense mind simply by their views and behaviour with regard to the 'Covid' hoax. We have had this division throughout known human history with the mass of the people on one side and individuals who could see and intuit beyond the walls of the simulation – Plato's prisoner who broke out of the cave and saw reality for what it is. Such people have always been targeted by Wetiko/Archon-possessed authority, burned at the stake or demonised as mad, bad and dangerous. The Cult today and its global network of 'anti-hate', 'anti-fascist' Woke groups are all expressions of Wetiko attacking those exposing the conspiracy, 'Covid' lies and the 'vaccine' agenda.

Woke as a whole is Wetiko which explains its black and white mentality and how at one it is with the Wetiko-possessed Cult. Paul Levy said: 'To be in this paradigm is to still be under the thrall of a two-valued logic – where things are either true or false – of a

wetikoized mind.' Wetiko consciousness is in a permanent rage, therefore so is Woke, and then there is Woke inversion and contradiction. 'Anti-fascists' act like fascists because fascists *and* 'anti-fascists' are both Wetiko at work. Political parties act the same while claiming to be different for the same reason. Secret society and satanic rituals are attaching initiates to Wetiko and the cold, ruthless, psychopathic mentality that secures the positions of power all over the world is Wetiko. Reframing 'training programmes' have the same cumulative effect of attaching Wetiko and we have their graduates described as automatons and robots with a cold, psychopathic, uncaring demeanour. They are all traits of Wetiko possession and look how many times they have been described in this book and elsewhere with regard to personnel behind 'Covid' including the police and medical profession. Climbing the greasy pole in any profession in a Wetiko society requires traits of Wetiko to get there and that is particularly true of politics which is not about fair competition and pre-eminence of ideas. It is founded on how many backs you can stab and arses you can lick. This culminated in the global 'Covid' coordination between the Wetiko possessed who pulled it off in all the different countries without a trace of empathy and compassion for their impact on humans. Our sight sense can see only holographic form and not the Field which connects holographic form. Therefore we perceive 'physical' objects with 'space' in between. In fact that 'space' is energy/consciousness operating on multiple frequencies. One of them is Wetiko and that connects the Cult psychopaths, those who submit to the psychopaths, and those who serve the psychopaths in the media operations of the world. Wetiko is Gates. Wetiko is the mask-wearing submissive. Wetiko is the fake journalist and 'fact-checker'. The Wetiko Field is coordinating the whole thing. Psychopaths, gofers, media operatives, 'anti-hate' hate groups, 'fact-checkers' and submissive people work as one unit *even without human coordination* because they are attached to the *same* Field which is organising it all (Fig 22). Paul Levy is here describing how Wetiko-possessed people are drawn together and refuse to let any information breach their rigid

perceptions. He was writing long before 'Covid', but I think you will recognise followers of the 'Covid' religion *oh just a little bit*:

People who are channelling the vibratory frequency of wetiko align with each other through psychic resonance to reinforce their unspoken shared agreement so as to uphold their deranged view of reality. Once an unconscious content takes possession of certain individuals, it irresistibly draws them together by mutual attraction and knits them into groups tied together by their shared madness that can easily swell into an avalanche of insanity.

A psychic epidemic is a closed system, which is to say that it is insular and not open to any new information or informing influences from the outside world which contradict its fixed, limited, and limiting perspective.

There we have the Woke mind and the 'Covid' mind. Compatible resonance draws the awakening together, too, which is clearly happening today.

Figure 22: The Wetiko Field from which the Cult pyramid and its personnel are made manifest. (Image by Neil Hague).

Spiritual servitude

Wetiko doesn't care about humans. It's not human; it just possesses humans for its own ends and the effect (depending on the scale of

possession) can be anything from extreme psychopathy to unquestioning obedience. Wetiko's worst nightmare is for human consciousness to expand beyond the simulation. Everything is focussed on stopping that happening through control of information, thus perception, thus frequency. The 'education system', media, science, medicine, academia, are all geared to maintaining humanity in five-sense servitude as is the constant stimulation of low-vibrational mental and emotional states (see 'Covid'). Wetiko seeks to dominate those subconscious spaces between five-sense perception and expanded consciousness where the computer meets the operator. From these subconscious hiding places Wetiko speaks to us to trigger urges and desires that we take to be our own and manipulate us into anything from low-vibrational to psychopathic states. Remember how Islam describes the Jinn as invisible tricksters that 'whisper' and confuse. Wetiko is the origin of the 'trickster god' theme that you find in cultures all over the world. Jinn, like the Archons, are Wetiko which is terrified of humans awakening and reconnecting with our true self for then its energy source has gone. With that the feedback loop breaks between Wetiko and human perception that provides the energetic momentum on which its very existence depends as a force of evil. Humans are both its target and its source of survival, but only if we are operating in low-vibrational states of fear, hate, depression and the background anxiety that most people suffer. We are Wetiko's target because we are its key to survival. It needs us, not the other way round. Paul Levy writes:

A vampire has no intrinsic, independent, substantial existence in its own right; it only exists in relation to us. The pathogenic, vampiric mind-parasite called wetiko is nothing in itself – not being able to exist from its own side – yet it has a 'virtual reality' such that it can potentially destroy our species …

…The fact that a vampire is not reflected by a mirror can also mean that what we need to see is that there's nothing, no-thing to see, other than ourselves. The fact that wetiko is the expression of something inside of us means that the cure for wetiko is with us as well. The critical issue is finding this cure within us and then putting it into effect.

Evil begets evil because if evil does not constantly expand and find new sources of energetic sustenance its evil, its *distortion*, dies with the assimilation into balance and harmony. Love is the garlic to Wetiko's vampire. Evil, the absence of love, cannot exist in the presence of love. I think I see a way out of here. I have emphasised so many times over the decades that the Archons/Wetiko and their Cult are not all powerful. *They are not*. I don't care how it looks even now *they are not*. I have not called them little boys in short trousers for effect. I have said it because it is true. Wetiko's insatiable desire for power over others is not a sign of its omnipotence, but its insecurity. Paul Levy writes: 'Due to the primal fear which ultimately drives it and which it is driven to cultivate, wetiko's body politic has an intrinsic and insistent need for centralising power and control so as to create imagined safety for itself.' *Yeeeeeees!* Exactly! Why does Wetiko want humans in an ongoing state of fear? Wetiko itself *is* fear and it is petrified of love. As evil is an absence of love, so love is an absence of fear. Love conquers all and *especially* Wetiko which *is* fear. Wetiko brought fear into the world when it wasn't here before. *Fear* was the 'fall', the fall into low-frequency ignorance and illusion – fear is **F**alse **E**motion **A**ppearing **R**eal. The simulation is driven and energised by fear because Wetiko/Yaldabaoth (fear) *are* the simulation. Fear is the absence of love and Wetiko is the absence of love.

Wetiko today

We can now view current events from this level of perspective. The 'Covid' hoax has generated momentous amounts of ongoing fear, anxiety, depression and despair which have empowered Wetiko. No wonder people like Gates have been the instigators when they are Wetiko incarnate and exhibit every trait of Wetiko in the extreme. See how cold and unemotional these people are like Gates and his cronies, how dead of eye they are. That's Wetiko. Sabbatians are Wetiko and everything they control including the World Health Organization, Big Pharma and the 'vaccine' makers, national 'health'

hierarchies, corporate media, Silicon Valley, the banking system, and the United Nations with its planned transformation into world government. All are controlled and possessed by the Wetiko distortion into distorting human society in its image. We are with this knowledge at the gateway to understanding the world. Divisions of race, culture, creed and sexuality are diversions to hide the real division between those possessed and influenced by Wetiko and those that are not. The 'Covid' hoax has brought both clearly into view. Human behaviour is not about race. Tyrants and dictatorships come in all colours and creeds. What unites the US president bombing the innocent and an African tribe committing genocide against another as in Rwanda? What unites them? *Wetiko*. All wars are Wetiko, all genocide is Wetiko, all hunger over centuries in a world of plenty is Wetiko. Children going to bed hungry, including in the West, is Wetiko. Cult-generated Woke racial divisions that focus on the body are designed to obscure the reality that divisions in behaviour are manifestations of mind, not body. Obsession with body identity and group judgement is a means to divert attention from the real source of behaviour – mind and perception. Conflict sown by the Woke both within themselves and with their target groups are Wetiko providing lunch for itself through still more agents of the division, chaos, and fear on which it feeds. The Cult is seeking to assimilate the entirety of humanity and all children and young people into the Wetiko frequency by manipulating them into states of fear and despair. Witness all the suicide and psychological unravelling since the spring of 2020. Wetiko psychopaths want to impose a state of unquestioning obedience to authority which is no more than a conduit for Wetiko to enforce its will and assimilate humanity into itself. It needs us to believe that resistance is futile when it fears resistance and even more so the game-changing non-cooperation with its impositions. It can use violent resistance for its benefit. Violent impositions and violent resistance are *both* Wetiko. The Power of Love with its Power of No will sweep Wetiko from our world. Wetiko and its Cult know that. They just don't want us to know.

AI Wetiko

This brings me to AI or artificial intelligence and something else Wetikos don't want us to know. What is AI *really*? I know about computer code algorithms and AI that learns from data input. These, however, are more diversions, the expeditionary force, for the real AI that they want to connect to the human brain as promoted by Silicon Valley Wetikos like Kurzweil. What is this AI? It is the frequency of *Wetiko*, the frequency of the Archons. The connection of AI to the human brain is the connection of the Wetiko frequency to create a Wetiko hive mind and complete the job of assimilation. The hive mind is planned to be controlled from Israel and China which are both 100 percent owned by Wetiko Sabbatians. The assimilation process has been going on minute by minute in the 'smart' era which fused with the 'Covid' era. We are told that social media is scrambling the minds of the young and changing their personality. This is true, but what is social media? Look more deeply at how it works, how it creates divisions and conflict, the hostility and cruelty, the targeting of people until they are destroyed. That's Wetiko. Social media is manipulated to tune people to the Wetiko frequency with all the emotional exploitation tricks employed by platforms like Facebook and its Wetiko front man, Zuckerberg. Facebook's Instagram announced a new platform for children to overcome a legal bar on them using the main site. This is more Wetiko exploitation and manipulation of kids. Amnesty International likened the plan to foxes offering to guard the henhouse and said it was incompatible with human rights. Since when did Wetiko or Zuckerberg (I repeat myself) care about that? Would Brin and Page at Google, Wojcicki at YouTube, Bezos at Amazon and whoever the hell runs Twitter act as they do if they were not channelling Wetiko? Would those who are developing technologies for no other reason than human control? How about those designing and selling technologies to kill people and Big Pharma drug and 'vaccine' producers who know they will end or devastate lives? Quite a thought for these people to consider is that if you are Wetiko in a human life you are Wetiko on the 'other side' unless your frequency

changes and that can only change by a change of perception which becomes a change of behaviour. Where Gates is going does not bear thinking about although perhaps that's exactly where he wants to go. Either way, that's where he's going. His frequency will make it so.

The frequency lair

I have been saying for a long time that a big part of the addiction to smartphones and devices is that a frequency is coming off them that entraps the mind. People spend ages on their phones and sometimes even a minute or so after they put them down they pick them up again and it all repeats. 'Covid' lockdowns will have increased this addiction a million times for obvious reasons. Addictions to alcohol overindulgence and drugs are another way that Wetiko entraps consciousness to attach to its own. Both are symptoms of low-vibrational psychological distress which alcoholism and drug addiction further compound. Do we think it's really a coincidence that access to them is made so easy while potions that can take people into realms beyond the simulation are banned and illegal? I have explored smartphone addiction in other books, the scale is mind-blowing, and that level of addiction does not come without help. Tech companies that make these phones are Wetiko and they will have no qualms about destroying the minds of children. We are seeing again with these companies the Wetiko perceptual combination of psychopathic enforcers and weak and meek unquestioning compliance by the rank and file.

The global Smart Grid is the Wetiko Grid and it is crucial to complete the Cult endgame. The simulation is radiation and we are being deluged with technological radiation on a devastating scale. Wetiko frauds like Elon Musk serve Cult interests while occasionally criticising them to maintain his street-cred. 5G and other forms of Wi-Fi are being directed at the earth from space on a volume and scale that goes on increasing by the day. Elon Musk's (officially) SpaceX Starlink project is in the process of putting tens of thousands of satellites in low orbit to cover every inch of the planet with 5G and other Wi-Fi to create Kurzweil's global 'cloud' to which the

human mind is planned to be attached very soon. SpaceX has approval to operate 12,000 satellites with more than 1,300 launched at the time of writing and applications filed for *30,000* more. Other operators in the Wi-Fi, 5G, low-orbit satellite market include OneWeb (UK), Telesat (Canada), and AST & Science (US). Musk tells us that AI could be the end of humanity and then launches a company called Neuralink to connect the human brain to computers. Musk's (in theory) Tesla company is building electric cars and the driverless vehicles of the smart control grid. As frauds and bullshitters go Elon Musk in my opinion is Major League.

5G and technological radiation in general are destructive to human health, genetics and psychology and increasing the strength of artificial radiation underpins the five-sense perceptual bubbles which are themselves expressions of radiation or electromagnetism. Freedom activist John Whitehead was so right with his 'databit by databit, we are building our own electronic concentration camps'. The Smart Grid and 5G is a means to control the human mind and infuse perceptual information into The Field to influence anyone in sync with its frequency. You can change perception and behaviour en masse if you can manipulate the population into those levels of frequency and this is happening all around us today. The arrogance of Musk and his fellow Cult operatives knows no bounds in the way that we see with Gates. Musk's satellites are so many in number already they are changing the night sky when viewed from Earth. The astronomy community has complained about this and they have seen nothing yet. Some consequences of Musk's Wetiko hubris include: Radiation; visible pollution of the night sky; interference with astronomy and meteorology; ground and water pollution from intensive use of increasingly many spaceports; accumulating space debris; continual deorbiting and burning up of aging satellites, polluting the atmosphere with toxic dust and smoke; and ever-increasing likelihood of collisions. A collective public open letter of complaint to Musk said:

> We are writing to you … because SpaceX is in process of surrounding the Earth with a network of thousands of satellites whose very purpose is to irradiate every square inch of the

Earth. SpaceX, like everyone else, is treating the radiation as if it were not there. As if the mitochondria in our cells do not depend on electrons moving undisturbed from the food we digest to the oxygen we breathe.

As if our nervous systems and our hearts are not subject to radio frequency interference like any piece of electronic equipment. As if the cancer, diabetes, and heart disease that now afflict a majority of the Earth's population are not metabolic diseases that result from interference with our cellular machinery. As if insects everywhere, and the birds and animals that eat them, are not starving to death as a result.

People like Musk and Gates believe in their limitless Wetiko arrogance that they can do whatever they like to the world because they own it. Consequences for humanity are irrelevant. It's absolutely time that we stopped taking this shit from these self-styled masters of the Earth when you consider where this is going.

Why is the Cult so anti-human?

I hear this question often: Why would they do this when it will affect them, too? Ah, but will it? Who is this *them*? Forget their bodies. They are just vehicles for Wetiko consciousness. When you break it all down to the foundations we are looking at a state of severely distorted consciousness targeting another state of consciousness for assimilation. The rest is detail. The simulation is the fly-trap in which unique sensations of the five senses create a cycle of addiction called reincarnation. Renegade Minds see that everything which happens in our reality is a smaller version of the whole picture in line with the holographic principle. Addiction to the radiation of smart technology is a smaller version of addiction to the whole simulation. Connecting the body/brain to AI is taking that addiction on a giant step further to total ongoing control by assimilating human incarnate consciousness into Wetiko. I have watched during the 'Covid' hoax how many are becoming ever more profoundly attached to Wetiko's perceptual calling cards of aggressive response to any other point of view ('There is no other god but me'), psychopathic lack of compassion and empathy, and servile submission to the narrative and will of authority. Wetiko is the psychopaths *and* subservience to psychopaths. The Cult of Wetiko is

so anti-human because it is *not* human. It embarked on a mission to destroy human by targeting everything that it means to be human and to survive as human. 'Covid' is not the end, just a means to an end. The Cult with its Wetiko consciousness is seeking to change Earth systems, including the atmosphere, to suit them, not humans. The gathering bombardment of 5G alone from ground and space is dramatically changing The Field with which the five senses interact. There is so much more to come if we sit on our hands and hope it will all go away. It is not meant to go away. It is meant to get ever more extreme and we need to face that while we still can – just.

Carbon dioxide is the gas of life. Without that human is over. Kaput, gone, history. No natural world, no human. The Cult has created a cock and bull story about carbon dioxide and climate change to justify its reduction to the point where Gates and the ignoramus Biden 'climate chief' John Kerry want to suck it out of the atmosphere. Kerry wants to do this because his master Gates does. Wetikos have made the gas of life a demon with the usual support from the Wokers of Extinction Rebellion and similar organisations and the bewildered puppet-child that is Greta Thunberg who was put on the world stage by Klaus Schwab and the World Economic Forum. The name Extinction Rebellion is both ironic and as always Wetiko inversion. The gas that we need to survive must be reduced to save us from extinction. The most basic need of human is oxygen and we now have billions walking around in face nappies depriving body and brain of this essential requirement of human existence. More than that 5G at 60 gigahertz interacts with the oxygen molecule to reduce the amount of oxygen the body can absorb into the bloodstream. The obvious knock-on consequences of that for respiratory and cognitive problems and life itself need no further explanation. Psychopaths like Musk are assembling a global system of satellites to deluge the human atmosphere with this insanity. The man should be in jail. Here we have two most basic of human needs, oxygen and carbon dioxide, being dismantled.

Two others, water and food, are getting similar treatment with the United Nations Agendas 21 and 2030 – the Great Reset – planning to

centrally control all water and food supplies. People will not even own rain water that falls on their land. Food is affected at the most basic level by reducing carbon dioxide. We have genetic modification or GMO infiltrating the food chain on a mass scale, pesticides and herbicides polluting the air and destroying the soil. Freshwater fish that provide livelihoods for 60 million people and feed hundreds of millions worldwide are being 'pushed to the brink' according the conservationists while climate change is the only focus. Now we have Gates and Schwab wanting to dispense with current food sources all together and replace them with a synthetic version which the Wetiko Cult would control in terms of production and who eats and who doesn't. We have been on the Totalitarian Tiptoe to this for more than 60 years as food has become ever more processed and full of chemical shite to the point today when it's not natural food at all. As Dr Tom Cowan says: 'If it has a label don't eat it.' Bill Gates is now the biggest owner of farmland in the United States and he does nothing without an ulterior motive involving the Cult. Klaus Schwab wrote: 'To feed the world in the next 50 years we will need to produce as much food as was produced in the last 10,000 years … food security will only be achieved, however, if regulations on genetically modified foods are adapted to reflect the reality that gene editing offers a precise, efficient and safe method of improving crops.' Liar. People and the world are being targeted with aluminium through vaccines, chemtrails, food, drink cans, and endless other sources when aluminium has been linked to many health issues including dementia which is increasing year after year. Insects, bees and wildlife essential to the food chain are being deleted by pesticides, herbicides and radiation which 5G is dramatically increasing with 6G and 7G to come. The pollinating bee population is being devastated while wildlife including birds, dolphins and whales are having their natural radar blocked by the effects of ever-increasing radiation. In the summer windscreens used to be splattered with insects so numerous were they. It doesn't happen now. Where have they gone?

Synthetic everything

The Cult is introducing genetically-modified versions of trees, plants and insects including a Gates-funded project to unleash hundreds of millions of genetically-modified, lab-altered and patented male mosquitoes to mate with wild mosquitoes and induce genetic flaws that cause them to die out. Clinically-insane Gates-funded Japanese researchers have developed mosquitos that spread vaccine and are dubbed 'flying vaccinators'. Gates is funding the modification of weather patterns in part to sell the myth that this is caused by carbon dioxide and he's funding geoengineering of the skies to change the atmosphere. Some of this came to light with the Gates-backed plan to release tonnes of chalk into the atmosphere to 'deflect the Sun and cool the planet'. Funny how they do this while the heating effect of the Sun is not factored into climate projections focussed on carbon dioxide. The reason is that they want to reduce carbon dioxide (so don't mention the Sun), but at the same time they do want to reduce the impact of the Sun which is so essential to human life and health. I have mentioned the sun-cholesterol-vitamin D connection as they demonise the Sun with warnings about skin cancer (caused by the chemicals in sun cream they tell you to splash on). They come from the other end of the process with statin drugs to reduce cholesterol that turns sunlight into vitamin D. A lack of vitamin D leads to a long list of health effects and how vitamin D levels must have fallen with people confined to their homes over 'Covid'. Gates is funding other forms of geoengineering and most importantly chemtrails which are dropping heavy metals, aluminium and self-replicating nanotechnology onto the Earth which is killing the natural world. See *Everything You Need To Know, But Have Never Been Told* for the detailed background to this.

Every human system is being targeted for deletion by a force that's not human. The Wetiko Cult has embarked on the process of transforming the human body from biological to synthetic biological as I have explained. Biological is being replaced by the artificial and synthetic – Archontic 'countermimicry' – right across human society. The plan eventually is to dispense with the human body altogether

and absorb human consciousness – which it wouldn't really be by then – into cyberspace (the simulation which is Wetiko/Yaldabaoth). Preparations for that are already happening if people would care to look. The alternative media rightly warns about globalism and 'the globalists', but this is far bigger than that and represents the end of the human race as we know it. The 'bad copy' of prime reality that Gnostics describe was a bad copy of harmony, wonder and beauty to start with before Wetiko/Yaldabaoth set out to change the simulated 'copy' into something very different. The process was slow to start with. Entrapped humans in the simulation timeline were not technologically aware and they had to be brought up to intellectual speed while being suppressed spiritually to the point where they could build their own prison while having no idea they were doing so. We have now reached that stage where technological intellect has the potential to destroy us and that's why events are moving so fast. Central American shaman Don Juan Matus said:

> Think for a moment, and tell me how you would explain the contradictions between the intelligence of man the engineer and the stupidity of his systems of belief, or the stupidity of his contradictory behaviour. Sorcerers believe that the predators have given us our systems of beliefs, our ideas of good and evil; our social mores. They are the ones who set up our dreams of success or failure. They have given us covetousness, greed, and cowardice. It is the predator who makes us complacent, routinary, and egomaniacal.
>
> In order to keep us obedient and meek and weak, the predators engaged themselves in a stupendous manoeuvre – stupendous, of course, from the point of view of a fighting strategist; a horrendous manoeuvre from the point of those who suffer it. They gave us their mind. The predators' mind is baroque, contradictory, morose, filled with the fear of being discovered any minute now.

For 'predators' see Wetiko, Archons, Yaldabaoth, Jinn, and all the other versions of the same phenomenon in cultures and religions all over the world. The theme is always the same because it's true and it's real. We have reached the point where we have to deal with it. The question is – how?

Don't fight – walk away

I thought I'd use a controversial subheading to get things moving in terms of our response to global fascism. What do you mean 'don't fight'? What do you mean 'walk away'? We've got to fight. We can't walk away. Well, it depends what we mean by fight and walk away. If fighting means physical combat we are playing Wetiko's game and falling for its trap. It wants us to get angry, aggressive, and direct hate and hostility at the enemy we think we must fight. Every war, every battle, every conflict, has been fought with Wetiko leading both sides. It's what it does. Wetiko wants a fight, anywhere, any place. Just hit me, son, so I can hit you back. Wetiko hits Wetiko and Wetiko hits Wetiko in return. I am very forthright as you can see in exposing Wetikos of the Cult, but I don't hate them. I refuse to hate them. It's what they want. What you hate you become. What you *fight* you become. Wokers, 'anti-haters' and 'anti-fascists' prove this every time they reach for their keyboards or don their balaclavas. By walk away I mean to disengage from Wetiko which includes ceasing to cooperate with its tyranny. Paul Levy says of Wetiko:

> The way to 'defeat' evil is not to try to destroy it (for then, in playing evil's game, we have already lost), but rather, to find the invulnerable place within ourselves where evil is unable to vanquish us – this is to truly 'win' our battle with evil.

Wetiko is everywhere in human society and it's been on steroids since the 'Covid' hoax. Every shouting match over wearing masks has Wetiko wearing a mask and Wetiko not wearing one. It's an electrical circuit of push and resist, push and resist, with Wetiko pushing *and* resisting. Each polarity is Wetiko empowering itself. Dictionary definitions of 'resist' include 'opposing, refusing to accept or comply with' and the word to focus on is 'opposing'. What form does this take – setting police cars alight or 'refusing to accept or comply with'? The former is Wetiko opposing Wetiko while the other points the way forward. This is the difference between those aggressively demanding that government fascism must be obeyed who stand in stark contrast to the great majority of Pushbackers. We saw this clearly with a march by thousands of Pushbackers against lockdown in London followed days later by a Woker-hijacked

protest in Bristol in which police cars were set on fire. Masks were virtually absent in London and widespread in Bristol. Wetiko wants lockdown on every level of society and infuses its aggression to police it through its unknowing stooges. Lockdown protesters are the ones with the smiling faces and the hugs, The two blatantly obvious states of being – getting more obvious by the day – are the result of Wokers and their like becoming ever more influenced by the simulation Field of Wetiko and Pushbackers ever more influenced by The Field of a far higher vibration beyond the simulation. Wetiko can't invade the heart which is where most lockdown opponents are coming from. It's the heart that allows them to see through the lies to the truth in ways I will be highlighting.

Renegade Minds know that calmness is the place from which wisdom comes. You won't find wisdom in a hissing fit and wisdom is what we need in abundance right now. Calmness is not weakness – you don't have to scream at the top of your voice to be strong. Calmness is indeed a sign of strength. 'No' means I'm not doing it. *NOOOO!!!* doesn't mean you're not doing it even more. Volume does not advance 'No – I'm not doing it'. You are just not doing it. Wetiko possessed and influenced don't know how to deal with that. Wetiko wants a fight and we should not give it one. What it needs more than anything is our *cooperation* and we should not give that either. Mass rallies and marches are great in that they are a visual representation of feeling, but if it ends there they are irrelevant. You demand that Wetikos act differently? Well, they're not going to are they? They are Wetikos. We don't need to waste our time demanding that something doesn't happen when that will make no difference. We need to delete the means that *allows* it to happen. This, invariably, is our cooperation. You can demand a child stop firing a peashooter at the dog or you can refuse to buy the peashooter. If you provide the means you are cooperating with the dog being smacked on the nose with a pea. How can the authorities enforce mask-wearing if millions in a country refuse? What if the 74 million Pushbackers that voted for Trump in 2020 refused to wear masks, close their businesses or stay in their homes. It would be unenforceable. The

few control the many through the compliance of the many and that's always been the dynamic be it 'Covid' regulations or the Roman Empire. I know people can find it intimidating to say no to authority or stand out in a crowd for being the only one with a face on display; but it has to be done or it's over. I hope I've made clear in this book that where this is going will be far more intimidating than standing up now and saying 'No' – I will not cooperate with my own enslavement and that of my children. There might be consequences for some initially, although not so if enough do the same. The question that must be addressed is what is going to happen if we don't? It is time to be strong and unyieldingly so. No means no. Not here and there, but *everywhere* and *always*. I have refused to wear a mask and obey all the other nonsense. I will not comply with tyranny. I repeat: Fascism is not imposed by fascists – there are never enough of them. Fascism is imposed by the population acquiescing to fascism. *I will not do it*. I will die first, or my body will. Living meekly under fascism is a form of death anyway, the death of the spirit that Martin Luther King described.

Making things happen

We must not despair. This is not over till it's over and it's far from that. The 'fat lady' must refuse to sing. The longer the 'Covid' hoax has dragged on and impacted on more lives we have seen an awakening of phenomenal numbers of people worldwide to the realisation that what they have believed all their lives is not how the world really is. Research published by the system-serving University of Bristol and King's College London in February, 2021, concluded: 'One in every 11 people in Britain say they trust David Icke's take on the coronavirus pandemic.' It will be more by now and we have gathering numbers to build on. We must urgently progress from seeing the scam to ceasing to cooperate with it. Prominent German lawyer Reiner Fuellmich, also licenced to practice law in America, is doing a magnificent job taking the legal route to bring the psychopaths to justice through a second Nuremberg tribunal for crimes against humanity. Fuellmich has an impressive record of

beating the elite in court and he formed the German Corona Investigative Committee to pursue civil charges against the main perpetrators with a view to triggering criminal charges. Most importantly he has grasped the foundation of the hoax – the PCR test not testing for the 'virus' – and Christian Drosten is therefore on his charge sheet along with Gates frontman Tedros at the World Health Organization. Major players must be not be allowed to inflict their horrors on the human race without being brought to book. A life sentence must follow for Bill Gates and the rest of them. A group of researchers has also indicted the government of Norway for crimes against humanity with copies sent to the police and the International Criminal Court. The lawsuit cites participation in an internationally-planned false pandemic and violation of international law and human rights, the European Commission's definition of human rights by coercive rules, Nuremberg and Hague rules on fundamental human rights, and the Norwegian constitution. We must take the initiative from hereon and not just complain, protest and react.

There are practical ways to support vital mass non-cooperation. Organising in numbers is one. Lockdown marches in London in the spring in 2021 were mass non-cooperation that the authorities could not stop. There were too many people. Hundreds of thousands walked the London streets in the centre of the road for mile after mile while the Face-Nappies could only look on. They were determined, but calm, and just *did it* with no histrionics and lots of smiles. The police were impotent. Others are organising group shopping without masks for mutual support and imagine if that was happening all over. Policing it would be impossible. If the store refuses to serve people in these circumstances they would be faced with a long line of trolleys full of goods standing on their own and everything would have to be returned to the shelves. How would they cope with that if it kept happening? I am talking here about moving on from complaining to being pro-active; from watching things happen to making things happen. I include in this our relationship with the police. The behaviour of many Face-Nappies

has been disgraceful and anyone who thinks they would never find concentration camp guards in the 'enlightened' modern era have had that myth busted big-time. The period and setting may change – Wetikos never do. I watched film footage from a London march in which a police thug viciously kicked a protestor on the floor who had done nothing. His fellow Face-Nappies stood in a ring protecting him. What he did was a criminal assault and with a crowd far outnumbering the police this can no longer be allowed to happen unchallenged. I get it when people chant 'shame on you' in these circumstances, but that is no longer enough. They *have* no shame those who do this. Crowds needs to start making a citizen's arrest of the police who commit criminal offences and brutally attack innocent people and defenceless women. A citizen's arrest can be made under section 24A of the UK Police and Criminal Evidence (PACE) Act of 1984 and you will find something similar in other countries. I prefer to call it a Common Law arrest rather than citizen's for reasons I will come to shortly. Anyone can arrest a person committing an indictable offence or if they have reasonable grounds to suspect they are committing an indictable offence. On both counts the attack by the police thug would have fallen into this category. A citizen's arrest can be made to stop someone:

- Causing physical injury to himself or any other person
- Suffering physical injury
- Causing loss of or damage to property
- Making off before a constable can assume responsibility for him

A citizen's arrest may also be made to prevent a breach of the peace under Common Law and if they believe a breach of the peace will happen or anything related to harm likely to be done or already done in their presence. This is the way to go I think – the Common Law version. If police know that the crowd and members of the public will no longer be standing and watching while they commit

their thuggery and crimes they will think twice about acting like Brownshirts and Blackshirts.

Common Law – common sense

Mention of Common Law is very important. Most people think the law is the law as in one law. This is not the case. There are two bodies of law, Common Law and Statute Law, and they are not the same. Common Law is founded on the simple premise of do no harm. It does not recognise victimless crimes in which no harm is done while Statute Law does. There is a Statute Law against almost everything. So what is Statute Law? Amazingly it's the law of the *sea* that was brought ashore by the Cult to override the law of the land which is Common Law. They had no right to do this and as always they did it anyway. They had to. They could not impose their will on the people through Common Law which only applies to do no harm. How could you stitch up the fine detail of people's lives with that? Instead they took the law of the sea, or Admiralty Law, and applied it to the population. Statute Law refers to all the laws spewing out of governments and their agencies including all the fascist laws and regulations relating to 'Covid'. The key point to make is that Statute Law is *contract law*. It only applies between *contracting* corporations. Most police officers don't even know this. They have to be kept in the dark, too. Long ago when merchants and their sailing ships began to trade with different countries a contractual law was developed called Admiralty Law and other names. Again it only applied to *contracts* agreed between *corporate* entities. If there is no agreed contract the law of the sea had no jurisdiction *and that still applies to its new alias of Statute Law*. The problem for the Cult when the law of the sea was brought ashore was an obvious one. People were not corporations and neither were government entities. To overcome the latter they made governments and all associated organisations corporations. All the institutions are *private corporations* and I mean governments and their agencies, local councils, police, courts, military, US states, the whole lot. Go to the

Dun and Bradstreet corporate listings website for confirmation that they are all corporations. You are arrested by a private corporation called the police by someone who is really a private security guard and they take you to court which is another private corporation. Neither have jurisdiction over you unless you consent and *contract* with them. This is why you hear the mantra about law enforcement policing by *consent* of the people. In truth the people 'consent' only in theory through monumental trickery.

Okay, the Cult overcame the corporate law problem by making governments and institutions corporate entities; but what about people? They are not corporations are they? Ah ... well in a sense, and *only* a sense, they are. Not people exactly – the illusion of people. The Cult creates a corporation in the name of everyone at the time that their birth certificate is issued. Note birth/ *berth* certificate and when you go to court under the law of the sea on land you stand in a *dock*. These are throwbacks to the origin. My Common Law name is David Vaughan Icke. The name of the corporation created by the government when I was born is called Mr David Vaughan Icke usually written in capitals as MR DAVID VAUGHAN ICKE. That is not me, the living, breathing man. It is a fictitious corporate entity. The trick is to make you think that David Vaughan Icke and MR DAVID VAUGHAN ICKE are the same thing. *They are not*. When police charge you and take you to court they are prosecuting the corporate entity and not the living, breathing, man or woman. They have to trick you into identifying as the corporate entity and contracting with them. Otherwise they have no jurisdiction. They do this through a language known as legalese. Lawful and legal are not the same either. Lawful relates to Common Law and legal relates to Statute Law. Legalese is the language of Statue Law which uses terms that mean one thing to the public and another in legalese. Notice that when a police officer tells someone why they are being charged he or she will say at the end: 'Do you understand?' To the public that means 'Do you comprehend?' In legalese it means 'Do you stand under me?' Do you stand under my authority? If you say

yes to the question you are unknowingly agreeing to give them jurisdiction over you in a contract between two corporate entities.

This is a confidence trick in every way. Contracts have to be agreed between informed parties and if you don't know that David Vaughan Icke is agreeing to be the corporation MR DAVID VAUGHAN ICKE you cannot knowingly agree to contract. They are deceiving you and another way they do this is to ask for proof of identity. You usually show them a driving licence or other document on which your corporate name is written. In doing so you are accepting that you are that corporate entity when you are not. Referring to yourself as a 'person' or 'citizen' is also identifying with your corporate fiction which is why I made the Common Law point about the citizen's arrest. If you are approached by a police officer you identify yourself immediately as a living, breathing, man or woman and say 'I do not consent, I do not contract with you and I do not understand' or stand under their authority. I have a Common Law birth certificate as a living man and these are available at no charge from commonlawcourt.com. Businesses registered under the Statute Law system means that its laws apply. There are, however, ways to run a business under Common Law. Remember all 'Covid' laws and regulations are Statute Law – the law of *contracts* and you do not have to contract. This doesn't mean that you can kill someone and get away with it. Common Law says do no harm and that applies to physical harm, financial harm etc. Police are employees of private corporations and there needs to be a new system of non-corporate Common Law constables operating outside the Statute Law system. If you go to davidicke.com and put Common Law into the search engine you will find videos that explain Common Law in much greater detail. It is definitely a road we should walk.

With all my heart

I have heard people say that we are in a spiritual war. I don't like the term 'war' with its Wetiko dynamic, but I know what they mean. Sweep aside all the bodily forms and we are in a situation in which two states of consciousness are seeking very different realities.

Wetiko wants upheaval, chaos, fear, suffering, conflict and control. The other wants love, peace, harmony, fairness and freedom. That's where we are. We should not fall for the idea that Wetiko is all-powerful and there's nothing we can do. Wetiko is not all-powerful. It's a joke, pathetic. It doesn't have to be, but it has made that choice for now. A handful of times over the years when I have felt the presence of its frequency I have allowed it to attach briefly so I could consciously observe its nature. The experience is not pleasant, the energy is heavy and dark, but the ease with which you can kick it back out the door shows that its real power is in persuading us that it has power. It's all a con. Wetiko is a con. It's a trickster and not a power that can control us if we unleash our own. The con is founded on manipulating humanity to give its power to Wetiko which recycles it back to present the illusion that it has power when its power is *ours* that we gave away. This happens on an energetic level and plays out in the world of the seen as humanity giving its power to Wetiko authority which uses that power to control the population when the power is only the power the population has handed over. How could it be any other way for billions to be controlled by a relative few? I have had experiences with people possessed by Wetiko and again you can kick its arse if you do it with an open heart. Oh yes – the *heart* which can transform the world of perceived 'matter'.

We are receiver-transmitters and processors of information, but what information and where from? Information is processed into perception in three main areas – the brain, the heart and the belly. These relate to thinking, knowing, and emotion. Wetiko wants us to be head and belly people which means we think within the confines of the Matrix simulation and low-vibrational emotional reaction scrambles balance and perception. A few minutes on social media and you see how emotion is the dominant force. Woke is all emotion and is therefore thought-free and fact-free. Our heart is something different. It *knows* while the head *thinks* and has to try to work it out because it doesn't know. The human energy field has seven prime vortexes which connect us with wider reality (Fig 23). Chakra means

'wheels of light' in the Sanskrit language of ancient India. The main ones are: The crown chakra on top of the head; brow (or 'third eye') chakra in the centre of the forehead; throat chakra; heart chakra in the centre of the chest; solar plexus chakra below the sternum; sacral chakra beneath the navel; and base chakra at the bottom of the spine. Each one has a particular function or functions. We feel anxiety and nervousness in the belly where the sacral chakra is located and this processes emotion that can affect the colon to give people 'the shits' or make them 'shit scared' when they are nervous. Chakras all play an important role, but the Mr and Mrs Big is the heart chakra which sits at the centre of the seven, above the chakras that connect us to the 'physical' and below those that connect with higher realms (or at least should). Here in the heart chakra we feel love, empathy and compassion – 'My heart goes out to you'. Those with closed hearts become literally 'heart-less' in their attitudes and behaviour (see Bill Gates). Native Americans portrayed Wetiko with what Paul Levy calls a 'frigid, icy heart, devoid of mercy' (see Bill Gates).

Figure 23: The chakra system which interpenetrates the human energy field. The heart chakra is the governor – or should be.

Wetiko trembles at the thought of heart energy which it cannot infiltrate. The frequency is too high. What it seeks to do instead is close the heart chakra vortex to block its perceptual and energetic influence. Psychopaths have 'hearts of stone' and emotionally-damaged people have 'heartache' and 'broken hearts'. The astonishing amount of heart disease is related to heart chakra

disruption with its fundamental connection to the 'physical' heart. Dr Tom Cowan has written an outstanding book challenging the belief that the heart is a pump and making the connection between the 'physical' and spiritual heart. Rudolph Steiner who was way ahead of his time said the same about the fallacy that the heart is a pump. *What*? The heart is not a pump? That's crazy, right? Everybody knows that. Read Cowan's *Human Heart, Cosmic Heart* and you will realise that the very idea of the heart as a pump is ridiculous when you see the evidence. How does blood in the feet so far from the heart get pumped horizontally up the body by the heart?? Cowan explains in the book the real reason why blood moves as it does. Our 'physical' heart is used to symbolise love when the source is really the heart vortex or spiritual heart which is our most powerful energetic connection to 'out there' expanded consciousness. That's why we feel *knowing* – intuitive knowing – in the centre of the chest. Knowing doesn't come from a process of thoughts leading to a conclusion. It is there in an instant all in one go. Our heart knows because of its connection to levels of awareness that *do* know. This is the meaning and source of intuition – intuitive *knowing*.

For the last more than 30 years of uncovering the global game and the nature of reality my heart has been my constant antenna for truth and accuracy. An American intelligence insider once said that I had quoted a disinformer in one of my books and yet I had only quoted the part that was true. He asked: 'How do you do that?' By using my heart antenna was the answer and anyone can do it. Heart-centred is how we are meant to be. With a closed heart chakra we withdraw into a closed mind and the bubble of five-sense reality. If you take a moment to focus your attention on the centre of your chest, picture a spinning wheel of light and see it opening and expanding. You will feel it happening, too, and perceptions of the heart like joy and love as the heart impacts on the mind as they interact. The more the chakra opens the more you will feel expressions of heart consciousness and as the process continues, and becomes part of you, insights and knowings will follow. An open

heart is connected to that level of awareness that knows all is *One*. You will see from its perspective that the fault-lines that divide us are only illusions to control us. An open heart does not process the illusions of race, creed and sexuality except as brief experiences for a consciousness that is all. Our heart does not see division, only unity (Figs 24 and 25). There's something else, too. Our hearts love to laugh. Mark Twain's quote that says 'The human race has one really effective weapon, and that is laughter' is really a reference to the heart which loves to laugh with the joy of knowing the true nature of infinite reality and that all the madness of human society is an illusion of the mind. Twain also said: 'Against the assault of laughter nothing can stand.' This is so true of Wetiko and the Cult. Their insecurity demands that they be taken seriously and their power and authority acknowledged and feared. We should do nothing of the sort. We should not get aggressive or fearful which their insecurity so desires. We should laugh in their face. Even in their no-face as police come over in their face-nappies and expect to be taken seriously. They don't take themselves seriously looking like that so why should we? Laugh in the face of intimidation. Laugh in the face of tyranny. You will see by its reaction that you have pressed all of its buttons. Wetiko does not know what to do in the face of laughter or when its targets refuse to concede their joy to fear. We have seen many examples during the 'Covid' hoax when people have expressed their energetic power and the string puppets of Wetiko retreat with their tail limp between their knees. Laugh – the world is bloody mad after all and if it's a choice between laughter and tears I know which way I'm going.

Figure 24: Head consciousness without the heart sees division and everything apart from everything else.

Figure 25: Heart consciousness sees everything as One.

'Vaccines' and the soul

The foundation of Wetiko/Archon control of humans is the separation of incarnate five-sense mind from the infinite 'I' and closing the heart chakra where the True 'I' lives during a human life. The goal has been to achieve complete separation in both cases. I was interested therefore to read an account by a French energetic healer of what she said she experienced with a patient who had been given the 'Covid' vaccine. Genuine energy healers can sense information and consciousness fields at different levels of being which are referred to as 'subtle bodies'. She described treating the patient who later returned after having, without the healer's knowledge, two doses of the 'Covid vaccine'. The healer said:

I noticed immediately the change, very heavy energy emanating from [the] subtle bodies. The scariest thing was when I was working on the heart chakra, I connected with her soul: it was detached from the physical body, it had no contact and it was, as if it was floating in a state of total confusion: a damage to the consciousness that loses contact with the physical body, i.e. with our biological machine, there is no longer any communication between them.

I continued the treatment by sending light to the heart chakra, the soul of the person, but it seemed that the soul could no longer receive any light, frequency or energy. It was a very powerful experience for me. Then I understood that this substance is indeed used to detach consciousness so that this consciousness can no longer interact through this body that it possesses in life, where there is no longer any contact, no frequency, no light, no more energetic balance or mind.

This would create a human that is rudderless and at the extreme almost zombie-like operating with a fractional state of consciousness at the mercy of Wetiko. I was especially intrigued by what the healer said in the light of the prediction by the highly-informed Rudolf Steiner more than a hundred years ago. He said:

In the future, we will eliminate the soul with medicine. Under the pretext of a 'healthy point of view', there will be a vaccine by which the human body will be treated as soon as possible directly at birth, so that the human being cannot develop the thought of the existence of soul and Spirit. To materialistic doctors will be entrusted the task of removing the soul of humanity.

As today, people are vaccinated against this disease or that disease, so in the future, children will be vaccinated with a substance that can be produced precisely in such a way that people, thanks to this vaccination, will be immune to being subjected to the 'madness' of spiritual life. He would be extremely smart, but he would not develop a conscience, and that is the true goal of some materialistic circles.

Steiner said the vaccine would detach the physical body from the etheric body (subtle bodies) and 'once the etheric body is detached the relationship between the universe and the etheric body would become extremely unstable, and man would become an automaton'. He said 'the physical body of man must be polished on this Earth by spiritual will – so the vaccine becomes a kind of arymanique (Wetiko) force' and 'man can no longer get rid of a given materialistic feeling'. Humans would then, he said, become 'materialistic of constitution and can no longer rise to the spiritual'. I have been writing for years about DNA being a receiver-transmitter of information that connects us to other levels of reality and these 'vaccines' changing DNA can be likened to changing an antenna and what it can transmit and receive. Such a disconnection would clearly lead to changes in personality and perception. Steiner further predicted the arrival of AI. Big Pharma 'Covid vaccine' makers, expressions of Wetiko, are testing their DNA-manipulating evil on children as I write with a view to giving the 'vaccine' to babies. If it's a soul-body disconnector – and I say that it is or can be – every child would be disconnected from 'soul' at birth and the 'vaccine' would create a closed system in which spiritual guidance from the greater self would play no part. This has been the ambition of Wetiko all

along. A Pentagon video from 2005 was leaked of a presentation explaining the development of vaccines to change behaviour by their effect on the brain. Those that believe this is not happening with the 'Covid' genetically-modifying procedure masquerading as a 'vaccine' should make an urgent appointment with Naivety Anonymous. Klaus Schwab wrote in 2018:

> Neurotechnologies enable us to better influence consciousness and thought and to understand many activities of the brain. They include decoding what we are thinking in fine levels of detail through new chemicals and interventions that can influence our brains to correct for errors or enhance functionality.

The plan is clear and only the heart can stop it. With every heart that opens, every mind that awakens, Wetiko is weakened. Heart and love are far more powerful than head and hate and so nothing like a majority is needed to turn this around.

Beyond the Phantom

Our heart is the prime target of Wetiko and so it must be the answer to Wetiko. We *are* our heart which is part of one heart, the infinite heart. Our heart is where the true self lives in a human life behind firewalls of five-sense illusion when an imposter takes its place – *Phantom Self*; but our heart waits patiently to be set free any time we choose to see beyond the Phantom, beyond Wetiko. A Wetikoed Phantom Self can wreak mass death and destruction while the love of forever is locked away in its heart. The time is here to unleash its power and let it sweep away the fear and despair that is Wetiko. Heart consciousness does not seek manipulated, censored, advantage for its belief or religion, its activism and desires. As an expression of the One it treats all as One with the same rights to freedom and opinion. Our heart demands fairness for itself no more than for others. From this unity of heart we can come together in mutual support and transform this Wetikoed world into what reality is meant to be – a place of love, joy, happiness, fairness, justice and freedom. Wetiko has another agenda and that's why the world is as

it is, but enough of this nonsense. Wetiko can't stay where hearts are open and it works so hard to keep them closed. Fear is its currency and its food source and love in its true sense has no fear. Why would love have fear when it knows it is *All That Is, Has Been, And Ever Can Be* on an eternal exploration of all possibility? Love in this true sense is not the physical attraction that passes for love. This can be an expression of it, yes, but Infinite Love, a love without condition, goes far deeper to the core of all being. It *is* the core of all being. Infinite realty was born from love beyond the illusions of the simulation. Love infinitely expressed is the knowing that all is One and the swiftly-passing experience of separation is a temporary hallucination. You cannot disconnect from Oneness; you can only *perceive* that you have and withdraw from its influence. This is the most important of all perception trickery by the mind parasite that is Wetiko and the foundation of all its potential for manipulation.

If we open our hearts, open the sluice gates of the mind, and redefine self-identity amazing things start to happen. Consciousness expands or contracts in accordance with self-identity. When true self is recognised as infinite awareness and label self – Phantom Self – is seen as only a series of brief experiences life is transformed. Consciousness expands to the extent that self-identity expands and everything changes. You see unity, not division, the picture, not the pixels. From this we can play the long game. No more is an experience something in and of itself, but a fleeting moment in the eternity of forever. Suddenly people in uniform and dark suits are no longer intimidating. Doing what your heart knows to be right is no longer intimidating and consequences for those actions take on the same nature of a brief experience that passes in the blink of an infinite eye. Intimidation is all in the mind. Beyond the mind there is no intimidation.

An open heart does not consider consequences for what it knows to be right. To do so would be to consider not doing what it knows to be right and for a heart in its power that is never an option. The Renegade Mind is really the Renegade Heart. Consideration of consequences will always provide a getaway car for the mind and

the heart doesn't want one. What is right in the light of what we face today is to stop cooperating with Wetiko in all its forms and to do it without fear or compromise. You cannot compromise with tyranny when tyranny always demands more until it has everything. Life is your perception and you are your destiny. Change your perception and you change your life. Change collective perception and we change the world.

Come on people … One human family, One heart, One goal … *FREEEEEEDOM*!

We must settle for nothing less.

Postscript

The big scare story as the book goes to press is the 'Indian' variant and the world is being deluged with propaganda about the 'Covid catastrophe' in India which mirrors in its lies and misrepresentations what happened in Italy before the first lockdown in 2020.

The *New York Post* published a picture of someone who had 'collapsed in the street from Covid' in India in April, 2021, which was actually taken during a gas leak in May, 2020. Same old, same old. Media articles in mid-February were asking why India had been so untouched by 'Covid' and then as their vaccine rollout gathered pace the alleged 'cases' began to rapidly increase. Indian 'Covid vaccine' maker Bharat Biotech was funded into existence by the Bill and Melinda Gates Foundation (the pair announced their divorce in May, 2021, which is a pity because they so deserve each other). The Indian 'Covid crisis' was ramped up by the media to terrify the world and prepare people for submission to still more restrictions. The scam that worked the first time was being repeated only with far more people seeing through the deceit. Davidicke.com and Ickonic.com have sought to tell the true story of what is happening by talking to people living through the Indian nightmare which has nothing to do with 'Covid'. We posted a letter from 'Alisha' in Pune who told a very different story to government and media mendacity. She said scenes of dying people and overwhelmed hospitals were designed to hide what was really happening – genocide and starvation. Alisha said that millions had already died of starvation during the ongoing lockdowns while government and media were lying and making it look like the 'virus':

Restaurants, shops, gyms, theatres, basically everything is shut. The cities are ghost towns. Even so-called 'essential' businesses are only open till 11am in the morning. You basically have just an hour to buy food and then your time is up.

Inter-state travel and even inter-district travel is banned. The cops wait at all major crossroads to question why you are traveling outdoors or to fine you if you are not wearing a mask.

The medical community here is also complicit in genocide, lying about hospitals being full and turning away people with genuine illnesses, who need immediate care. They have even created a shortage of oxygen cylinders.

This is the classic Cult modus operandi played out in every country. Alisha said that people who would not have a PCR test not testing for the 'virus' were being denied hospital treatment. She said the people hit hardest were migrant workers and those in rural areas. Most businesses employed migrant workers and with everything closed there were no jobs, no income and no food. As a result millions were dying of starvation or malnutrition. All this was happening under Prime Minister Narendra Modi, a 100-percent asset of the Cult, and it emphasises yet again the scale of pure anti-human evil we are dealing with. Australia banned its people from returning home from India with penalties for trying to do so of up to five years in jail and a fine of £37,000. The manufactured 'Covid' crisis in India was being prepared to justify further fascism in the West. Obvious connections could be seen between the Indian 'vaccine' programme and increased 'cases' and this became a common theme. The Seychelles, the most per capita 'Covid vaccinated' population in the world, went back into lockdown after a 'surge of cases'.

Long ago the truly evil Monsanto agricultural biotechnology corporation with its big connections to Bill Gates devastated Indian farming with genetically-modified crops. Human rights activist Gurcharan Singh highlighted the efforts by the Indian government to complete the job by destroying the food supply to hundreds of millions with 'Covid' lockdowns. He said that 415 million people at the bottom of the disgusting caste system (still going whatever they say) were below the poverty line and struggled to feed themselves every year. Now the government was imposing lockdown at just the

time to destroy the harvest. This deliberate policy was leading to mass starvation. People may reel back at the suggestion that a government would do that, but Wetiko-controlled 'leaders' are capable of any level of evil. In fact what is described in India is in the process of being instigated worldwide. The food chain and food supply are being targeted at every level to cause world hunger and thus control. Bill Gates is not the biggest owner of farmland in America for no reason and destroying access to food aids both the depopulation agenda and the plan for synthetic 'food' already being funded into existence by Gates. Add to this the coming hyper-inflation from the suicidal creation of fake 'money' in response to 'Covid' and the breakdown of container shipping systems and you have a cocktail that can only lead one way and is meant to. The Cult plan is to crash the entire system to 'build back better' with the Great Reset.

'Vaccine' transmission

Reports from all over the world continue to emerge of women suffering menstrual and fertility problems after having the fake 'vaccine' and of the non-'vaccinated' having similar problems when interacting with the 'vaccinated'. There are far too many for 'coincidence' to be credible. We've had menopausal women getting periods, others having periods stop or not stopping for weeks, passing clots, sometimes the lining of the uterus, breast irregularities, and miscarriages (which increased by 400 percent in parts of the United States). Non-'vaccinated' men and children have suffered blood clots and nose bleeding after interaction with the 'vaccinated'. Babies have died from the effects of breast milk from a 'vaccinated' mother. Awake doctors – the small minority – speculated on the cause of non-'vaccinated' suffering the same effects as the 'vaccinated'. Was it nanotechnology in the synthetic substance transmitting frequencies or was it a straight chemical bioweapon that was being transmitted between people? I am not saying that some kind of chemical transmission is not one possible answer, but the foundation of all that the Cult does is frequency and

this is fertile ground for understanding how transmission can happen. American doctor Carrie Madej, an internal medicine physician and osteopath, has been practicing for the last 20 years, teaching medical students, and she says attending different meetings where the agenda for humanity was discussed. Madej, who operates out of Georgia, did not dismiss other possible forms of transmission, but she focused on frequency in search of an explanation for transmission. She said the Moderna and Pfizer 'vaccines' contained nano-lipid particles as a key component. This was a brand new technology never before used on humanity. 'They're using a nanotechnology which is pretty much little tiny computer bits … nanobots or hydrogel.' Inside the 'vaccines' was 'this sci-fi kind of substance' which suppressed immune checkpoints to get into the cell. I referred to this earlier as the 'Trojan horse' technique that tricks the cell into opening a gateway for the self-replicating synthetic material and while the immune system is artificially suppressed the body has no defences. Madej said the substance served many purposes including an on-demand ability to 'deliver the payload' and using the nano 'computer bits' as biosensors in the body. 'It actually has the ability to accumulate data from your body, like your breathing, your respiration, thoughts, emotions, all kinds of things.'

She said the technology obviously has the ability to operate through Wi-Fi and transmit and receive energy, messages, frequencies or impulses. 'Just imagine you're getting this new substance in you and it can react to things all around you, the 5G, your smart device, your phones.' We had something completely foreign in the human body that had never been launched large scale at a time when we were seeing 5G going into schools and hospitals (plus the Musk satellites) and she believed the 'vaccine' transmission had something to do with this: '… if these people have this inside of them … it can act like an antenna and actually transmit it outwardly as well.' The synthetic substance produced its own voltage and so it could have that kind of effect. This fits with my own contention that the nano receiver-transmitters are designed to connect people to the

Smart Grid and break the receiver-transmitter connection to expanded consciousness. That would explain the French energy healer's experience of the disconnection of body from 'soul' with those who have had the 'vaccine'. The nanobots, self-replicating inside the body, would also transmit the synthetic frequency which could be picked up through close interaction by those who have not been 'vaccinated'. Madej speculated that perhaps it was 5G and increased levels of other radiation that was causing the symptoms directly although interestingly she said that non-'vaccinated' patients had shown improvement when they were away from the 'vaccinated' person they had interacted with. It must be remembered that you can control frequency and energy with your mind and you can consciously create energetic barriers or bubbles with the mind to stop damaging frequencies from penetrating your field. American paediatrician Dr Larry Palevsky said the 'vaccine' was not a 'vaccine' and was never designed to protect from a 'viral' infection. He called it 'a massive, brilliant propaganda of genocide' because they didn't have to inject everyone to get the result they wanted. He said the content of the jabs was able to infuse any material into the brain, heart, lungs, kidneys, liver, sperm and female productive system. 'This is genocide; this is a weapon of mass destruction.' At the same time American colleges were banning students from attending if they didn't have this life-changing and potentially life-ending 'vaccine'. Class action lawsuits must follow when the consequences of this college fascism come to light. As the book was going to press came reports about fertility effects on sperm in 'vaccinated' men which would absolutely fit with what I have been saying and hospitals continued to fill with 'vaccine' reactions. Another question is what about transmission via blood transfusions? The NHS has extended blood donation restrictions from seven days after a 'Covid vaccination' to 28 days after even a sore arm reaction.

I said in the spring of 2020 that the then touted 'Covid vaccine' would be ongoing each year like the flu jab. A year later Pfizer CEO, the appalling Albert Bourla, said people would 'likely' need a 'booster dose' of the 'vaccine' within 12 months of getting 'fully

vaccinated' and then a yearly shot. 'Variants will play a key role', he said confirming the point. Johnson & Johnson CEO Alex Gorsky also took time out from his 'vaccine' disaster to say that people may need to be vaccinated against 'Covid-19' each year. UK Health Secretary, the psychopath Matt Hancock, said additional 'boosters' would be available in the autumn of 2021. This is the trap of the 'vaccine passport'. The public will have to accept every last 'vaccine' they introduce, including for the fake 'variants', or it would cease to be valid. The only other way in some cases would be continuous testing with a test not testing for the 'virus' and what is on the swabs constantly pushed up your noise towards the brain every time?

'Vaccines' changing behaviour

I mentioned in the body of the book how I believed we would see gathering behaviour changes in the 'vaccinated' and I am already hearing such comments from the non-'vaccinated' describing behaviour changes in friends, loved ones and work colleagues. This will only increase as the self-replicating synthetic material and nanoparticles expand in body and brain. An article in the *Guardian* in 2016 detailed research at the University of Virginia in Charlottesville which developed a new method for controlling brain circuits associated with complex animal behaviour. The method, dubbed 'magnetogenetics', involves genetically-engineering a protein called ferritin, which stores and releases iron, to create a magnetised substance – 'Magneto' – that can activate specific groups of nerve cells from a distance. This is claimed to be an advance on other methods of brain activity manipulation known as optogenetics and chemogenetics (the Cult has been developing methods of brain control for a long time). The ferritin technique is said to be non-invasive and able to activate neurons 'rapidly and reversibly'. In other words, human thought and perception. The article said that earlier studies revealed how nerve cell proteins 'activated by heat and mechanical pressure can be genetically engineered so that they become sensitive to radio waves and magnetic fields, by attaching them to an iron-storing protein called ferritin, or to inorganic

paramagnetic particles'. Sensitive to radio waves and magnetic fields? You mean like 5G, 6G and 7G? This is the human-AI Smart Grid hive mind we are talking about. The *Guardian* article said:

> … the researchers injected Magneto into the striatum of freely behaving mice, a deep brain structure containing dopamine-producing neurons that are involved in reward and motivation, and then placed the animals into an apparatus split into magnetised and non-magnetised sections.
>
> Mice expressing Magneto spent far more time in the magnetised areas than mice that did not, because activation of the protein caused the striatal neurons expressing it to release dopamine, so that the mice found being in those areas rewarding. This shows that Magneto can remotely control the firing of neurons deep within the brain, and also control complex behaviours.

Make no mistake this basic methodology will be part of the 'Covid vaccine' cocktail and using magnetics to change brain function through electromagnetic field frequency activation. The Pentagon is developing a 'Covid vaccine' using ferritin. Magnetics would explain changes in behaviour and why videos are appearing across the Internet as I write showing how magnets stick to the skin at the point of the 'vaccine' shot. Once people take these 'vaccines' anything becomes possible in terms of brain function and illness which will be blamed on 'Covid-19' and 'variants'. Magnetic field manipulation would further explain why the non-'vaccinated' are reporting the same symptoms as the 'vaccinated' they interact with and why those symptoms are reported to decrease when not in their company. Interestingly 'Magneto', a 'mutant', is a character in the Marvel Comic *X-Men* stories with the ability to manipulate magnetic fields and he believes that mutants should fight back against their human oppressors by any means necessary. The character was born Erik Lehnsherr to a Jewish family in Germany.

Cult-controlled courts

The European Court of Human Rights opened the door for mandatory 'Covid-19 vaccines' across the continent when it ruled in a Czech Republic dispute over childhood immunisation that legally

enforced vaccination could be 'necessary in a democratic society'. The 17 judges decided that compulsory vaccinations did not breach human rights law. On the face of it the judgement was so inverted you gasp for air. If not having a vaccine infused into your body is not a human right then what is? Ah, but they said human rights law which has been specifically written to delete all human rights at the behest of the state (the Cult). Article 8 of the European Convention on Human Rights relates to the right to a private life. The crucial word here is *'except'*:

> There shall be no interference by a public authority with the exercise of this right EXCEPT such as is in accordance with the law and is necessary in a democratic society in the interests of national security, public safety or the economic wellbeing of the country, for the prevention of disorder or crime, for the protection of health or morals, or for the protection of the rights and freedoms of others [My emphasis].

No interference *except* in accordance with the law means there *are* no 'human rights' *except* what EU governments decide you can have at their behest. 'As is necessary in a democratic society' explains that reference in the judgement and 'in the interests of national security, public safety or the economic well-being of the country, for the prevention of disorder or crime, for the protection of health or morals, or for the protection of the rights and freedoms of others' gives the EU a coach and horses to ride through 'human rights' and scatter them in all directions. The judiciary is not a check and balance on government extremism; it is a vehicle to enforce it. This judgement was almost laughably predictable when the last thing the Cult wanted was a decision that went against mandatory vaccination. Judges rule over and over again to benefit the system of which they are a part. Vaccination disputes that come before them are invariably delivered in favour of doctors and authorities representing the view of the state which owns the judiciary. Oh, yes, and we have even had calls to stop putting 'Covid-19' on death certificates within 28 days of a 'positive test' because it is claimed the practice makes the 'vaccine' appear not to work. They are laughing at you.

The scale of madness, inhumanity and things to come was highlighted when those not 'vaccinated' for 'Covid' were refused evacuation from the Caribbean island of St Vincent during massive volcanic eruptions. Cruise ships taking residents to the safety of another island allowed only the 'vaccinated' to board and the rest were left to their fate. Even in life and death situations like this we see 'Covid' stripping people of their most basic human instincts and the insanity is even more extreme when you think that fake 'vaccine'-makers are not even claiming their body-manipulating concoctions stop 'infection' and 'transmission' of a 'virus' that doesn't exist. St Vincent Prime Minister Ralph Gonsalves said: 'The chief medical officer will be identifying the persons already vaccinated so that we can get them on the ship.' Note again the power of the chief medical officer who, like Whitty in the UK, will be answering to the World Health Organization. This is the Cult network structure that has overridden politicians who 'follow the science' which means doing what WHO-controlled 'medical officers' and 'science advisers' tell them. Gonsalves even said that residents who were 'vaccinated' after the order so they could board the ships would still be refused entry due to possible side effects such as 'wooziness in the head'. The good news is that if they were woozy enough in the head they could qualify to be prime minister of St Vincent.

Microchipping freedom

The European judgement will be used at some point to justify moves to enforce the 'Covid' DNA-manipulating procedure. Sandra Ro, CEO of the Global Blockchain Business Council, told a World Economic Forum event that she hoped 'vaccine passports' would help to 'drive forced consent and standardisation' of global digital identity schemes: 'I'm hoping with the desire and global demand for some sort of vaccine passport – so that people can get travelling and working again – [it] will drive forced consent, standardisation, and frankly, cooperation across the world.' The lady is either not very bright, or thoroughly mendacious, to use the term 'forced consent'.

You do not 'consent' if you are forced – you *submit*. She was describing what the plan has been all along and that's to enforce a digital identity on every human without which they could not function. 'Vaccine passports' are opening the door and are far from the end goal. A digital identity would allow you to be tracked in everything you do in cyberspace and this is the same technique used by Cult-owned China to enforce its social credit system of total control. The ultimate 'passport' is planned to be a microchip as my books have warned for nearly 30 years. Those nice people at the Pentagon working for the Cult-controlled Defense Advanced Research Projects Agency (DARPA) claimed in April, 2021, they have developed a microchip inserted under the skin to detect 'asymptomatic Covid-19 infection' before it becomes an outbreak and a 'revolutionary filter' that can remove the 'virus' from the blood when attached to a dialysis machine. The only problems with this are that the 'virus' does not exist and people transmitting the 'virus' with no symptoms is brain-numbing bullshit. This is, of course, not a ruse to get people to be microchipped for very different reasons. DARPA also said it was producing a one-stop 'vaccine' for the 'virus' and all 'variants'. One of the most sinister organisations on Planet Earth is doing this? Better have it then. These people are insane because Wetiko that possesses them is insane.

Researchers from the Salk Institute in California announced they have created an embryo that is part human and part monkey. My books going back to the 1990s have exposed experiments in top secret underground facilities in the United States where humans are being crossed with animal and non-human 'extraterrestrial' species. They are now easing that long-developed capability into the public arena and there is much more to come given we are dealing with psychiatric basket cases. Talking of which – Elon Musk's scientists at Neuralink trained a monkey to play Pong and other puzzles on a computer screen using a joystick and when the monkey made the correct move a metal tube squirted banana smoothie into his mouth which is the basic technique for training humans into unquestioning compliance. Two Neuralink chips were in the monkey's skull and

more than 2,000 wires 'fanned out' into its brain. Eventually the monkey played a video game purely with its brain waves. Psychopathic narcissist Musk said the 'breakthrough' was a step towards putting Neuralink chips into human skulls and merging minds with artificial intelligence. *Exactly*. This man is so dark and Cult to his DNA.

World Economic Fascism (WEF)

The World Economic Forum is telling you the plan by the statements made at its many and various events. Cult-owned fascist YouTube CEO Susan Wojcicki spoke at the 2021 WEF Global Technology Governance Summit (see the name) in which 40 governments and 150 companies met to ensure 'the responsible design and deployment of emerging technologies'. Orwellian translation: 'Ensuring the design and deployment of long-planned technologies will advance the Cult agenda for control and censorship.' Freedom-destroyer and Nuremberg-bound Wojcicki expressed support for tech platforms like hers to censor content that is 'technically legal but could be harmful'. Who decides what is 'harmful'? She does and they do. 'Harmful' will be whatever the Cult doesn't want people to see and we have legislation proposed by the UK government that would censor content on the basis of 'harm' no matter if the information is fair, legal and provably true. Make that *especially* if it is fair, legal and provably true. Wojcicki called for a global coalition to be formed to enforce content moderation standards through automated censorship. This is a woman and mega-censor so self-deluded that she shamelessly accepted a 'free expression' award – *Wojcicki* – in an event sponsored by her own *YouTube*. They have no shame and no self-awareness.

You know that 'Covid' is a scam and Wojcicki a Cult operative when YouTube is censoring medical and scientific opinion purely on the grounds of whether it supports or opposes the Cult 'Covid' narrative. Florida governor Ron DeSantis compiled an expert panel with four professors of medicine from Harvard, Oxford, and Stanford Universities who spoke against forcing children and

vaccinated people to wear masks. They also said there was no proof that lockdowns reduced spread or death rates of 'Covid-19'. Cult-gofer Wojcicki and her YouTube deleted the panel video 'because it included content that contradicts the consensus of local and global health authorities regarding the efficacy of masks to prevent the spread of Covid-19'. This 'consensus' refers to what the Cult tells the World Health Organization to say and the WHO tells 'local health authorities' to do. Wojcicki knows this, of course. The panellists pointed out that censorship of scientific debate was responsible for deaths from many causes, but Wojcicki couldn't care less. She would not dare go against what she is told and as a disgrace to humanity she wouldn't want to anyway. The UK government is seeking to pass a fascist 'Online Safety Bill' to specifically target with massive fines and other means non-censored video and social media platforms to make them censor 'lawful but harmful' content like the Cult-owned Facebook, Twitter, Google and YouTube. What is 'lawful but harmful' would be decided by the fascist Blair-created Ofcom.

Another WEF obsession is a cyber-attack on the financial system and this is clearly what the Cult has planned to take down the bank accounts of everyone – except theirs. Those that think they have enough money for the Cult agenda not to matter to them have got a big lesson coming if they continue to ignore what is staring them in the face. The World Economic Forum, funded by Gates and fronted by Klaus Schwab, announced it would be running a 'simulation' with the Russian government and global banks of just such an attack called Cyber Polygon 2021. What they simulate – as with the 'Covid' Event 201 – they plan to instigate. The WEF is involved in a project with the Cult-owned Carnegie Endowment for International Peace called the WEF-Carnegie Cyber Policy Initiative which seeks to merge Wall Street banks, 'regulators' (I love it) and intelligence agencies to 'prevent' (arrange and allow) a cyber-attack that would bring down the global financial system as long planned by those that control the WEF and the Carnegie operation. The Carnegie Endowment for International Peace sent an instruction to First World

War US President Woodrow Wilson not to let the war end before society had been irreversibly transformed.

The Wuhan lab diversion

As I close, the Cult-controlled authorities and lapdog media are systematically pushing 'the virus was released from the Wuhan lab' narrative. There are two versions – it happened by accident and it happened on purpose. Both are nonsense. The perceived existence of the never-shown-to-exist 'virus' is vital to sell the impression that there is actually an infective agent to deal with and to allow the endless potential for terrifying the population with 'variants' of a 'virus' that does not exist. The authorities at the time of writing are going with the 'by accident' while the alternative media is promoting the 'on purpose'. Cable news host Tucker Carlson who has questioned aspects of lockdown and 'vaccine' compulsion has bought the Wuhan lab story. 'Everyone now agrees' he said. Well, I don't and many others don't and the question is *why* does the system and its media suddenly 'agree'? When the media moves as one unit with a narrative it is always a lie – witness the hour by hour mendacity of the 'Covid' era. Why would this Cult-owned combination which has unleashed lies like machine gun fire suddenly 'agree' to tell the truth??

Much of the alternative media is buying the lie because it fits the conspiracy narrative, but it's the *wrong* conspiracy. The real conspiracy is that *there is no virus* and that is what the Cult is desperate to hide. The idea that the 'virus' was released by accident is ludicrous when the whole 'Covid' hoax was clearly long-planned and waiting to be played out as it was so fast in accordance with the Rockefeller document and Event 201. So they prepared everything in detail over decades and then sat around strumming their fingers waiting for an 'accidental' release from a bio-lab? *What??* It's crazy. Then there's the 'on purpose' claim. You want to circulate a 'deadly virus' and hide the fact that you've done so and you release it down the street from the highest-level bio-lab in China? I repeat – *What??*

You would release it far from that lab to stop any association being made. But, no, we'll do it in a place where the connection was certain to be made. Why would you need to scam 'cases' and 'deaths' and pay hospitals to diagnose 'Covid-19' if you had a real 'virus'? What are sections of the alternative media doing believing this crap? Where were all the mass deaths in Wuhan from a 'deadly pathogen' when the recovery to normal life after the initial propaganda was dramatic in speed? Why isn't the 'deadly pathogen' now circulating all over China with bodies in the street? Once again we have the technique of tell them what they want to hear and they will likely believe it. The alternative media has its 'conspiracy' and with Carlson it fits with his 'China is the danger' narrative over years. China *is* a danger as a global Cult operations centre, but not for this reason. The Wuhan lab story also has the potential to instigate conflict with China when at some stage the plan is to trigger a Problem-Reaction-Solution confrontation with the West. Question everything – *everything* – and especially when the media agrees on a common party line.

Third wave ... fourth wave ... fifth wave ...

As the book went into production the world was being set up for more lockdowns and a 'third wave' supported by invented 'variants' that were increasing all the time and will continue to do so in public statements and computer programs, but not in reality. India became the new Italy in the 'Covid' propaganda campaign and we were told to be frightened of the new 'Indian strain'. Somehow I couldn't find it within myself to do so. A document produced for the UK government entitled 'Summary of further modelling of easing of restrictions – Roadmap Step 2' declared that a third wave was inevitable (of course when it's in the script) and it would be the fault of children and those who refuse the health-destroying fake 'Covid vaccine'. One of the computer models involved came from the Cult-owned *Imperial College* and the other from Warwick University which I wouldn't trust to tell me the date in a calendar factory. The document states that both models presumed extremely high uptake

of the 'Covid vaccines' and didn't allow for 'variants'. The document states: 'The resurgence is a result of some people (mostly children) being ineligible for vaccination; others choosing not to receive the vaccine; and others being vaccinated but not perfectly protected.' The mendacity takes the breath away. Okay, blame those with a brain who won't take the DNA-modifying shots and put more pressure on children to have it as 'trials' were underway involving children as young as six months with parents who give insanity a bad name. Massive pressure is being put on the young to have the fake 'vaccine' and child age consent limits have been systematically lowered around the world to stop parents intervening. Most extraordinary about the document was its claim that the 'third wave' would be driven by 'the resurgence in both hospitalisations and deaths … dominated by *those that have received two doses of the vaccine*, comprising around 60-70% of the wave respectively'. The predicted peak of the 'third wave' suggested 300 deaths per day with 250 of them *fully 'vaccinated' people*. How many more lies do acquiescers need to be told before they see the obvious? Those who took the jab to 'protect themselves' are projected to be those who mostly get sick and die? So what's in the 'vaccine'? The document went on:

> It is possible that a summer of low prevalence could be followed by substantial increases in incidence over the following autumn and winter. Low prevalence in late summer should not be taken as an indication that SARS-CoV-2 has retreated or that the population has high enough levels of immunity to prevent another wave.

They are telling you the script and while many British people believed 'Covid' restrictions would end in the summer of 2021 the government was preparing for them to be ongoing. Authorities were awarding contracts for 'Covid marshals' to police the restrictions with contracts starting in July, 2021, and going through to January 31st, 2022, and the government was advertising for 'Media Buying Services' to secure media propaganda slots worth a potential £320 million for 'Covid-19 campaigns' with a contract not ending until March, 2022. The recipient – via a list of other front companies – was reported to be American media marketing giant Omnicom Group

Inc. While money is no object for 'Covid' the UK waiting list for all other treatment – including life-threatening conditions – passed 4.5 million. Meantime the Cult is seeking to control all official 'inquiries' to block revelations about what has really been happening and why. It must not be allowed to – we need Nuremberg jury trials in every country. The cover-up doesn't get more obvious than appointing ultra-Zionist professor Philip Zelikow to oversee two dozen US virologists, public health officials, clinicians, former government officials and four American 'charitable foundations' to 'learn the lessons' of the 'Covid' debacle. The personnel will be those that created and perpetuated the 'Covid' lies while Zelikow is the former executive director of the 9/11 Commission who ensured that the truth about those attacks never came out and produced a report that must be among the most mendacious and manipulative documents ever written – see *The Trigger* for the detailed exposure of the almost unimaginable 9/11 story in which Sabbatians can be found at every level.

Passive no more

People are increasingly challenging the authorities with amazing numbers of people taking to the streets in London well beyond the ability of the Face-Nappies to stop them. Instead the Nappies choose situations away from the mass crowds to target, intimidate, and seek to promote the impression of 'violent protestors'. One such incident happened in London's Hyde Park. Hundreds of thousands walking through the streets in protest against 'Covid' fascism were ignored by the Cult-owned BBC and most of the rest of the mainstream media, but they delighted in reporting how police were injured in 'clashes with protestors'. The truth was that a group of people gathered in Hyde Park at the end of one march when most had gone home and they were peacefully having a good time with music and chat. Face-Nappies who couldn't deal with the full-march crowd then waded in with their batons and got more than they bargained for. Instead of just standing for this criminal brutality the crowd used their numerical superiority to push the Face-Nappies out of the

park. Eventually the Nappies turned and ran. Unfortunately two or three idiots in the crowd threw drink cans striking two officers which gave the media and the government the image they wanted to discredit the 99.9999 percent who were peaceful. The idiots walked straight into the trap and we must always be aware of potential agent provocateurs used by the authorities to discredit their targets.

This response from the crowd – the can people apart – must be a turning point when the public no longer stand by while the innocent are arrested and brutally attacked by the Face-Nappies. That doesn't mean to be violent, that's the last thing we need. We'll leave the violence to the Face-Nappies and government. But it does mean that when the Face-Nappies use violence against peaceful people the numerical superiority is employed to stop them and make citizen's arrests or Common Law arrests for a breach of the peace. The time for being passive in the face of fascism is over.

We are the many, they are the few, and we need to make that count before there is no freedom left and our children and grandchildren face an ongoing fascist nightmare.

COME ON PEOPLE – IT'S TIME.

One final thought …

The power of love
A force from above
Cleaning my soul
Flame on burn desire
Love with tongues of fire
Purge the soul
Make love your goal

I'll protect you from the hooded claw
Keep the vampires from your door
When the chips are down I'll be around
With my undying, death-defying
Love for you

Envy will hurt itself
Let yourself be beautiful
Sparkling love, flowers
And pearls and pretty girls
Love is like an energy
Rushin' rushin' inside of me

This time we go sublime
Lovers entwine, divine, divine,
Love is danger, love is pleasure
Love is pure – the only treasure

I'm so in love with you
Purge the soul
Make love your goal

The power of love
A force from above
Cleaning my soul
The power of love
A force from above
A sky-scraping dove

Flame on burn desire
Love with tongues of fire
Purge the soul
Make love your goal

Frankie Goes To Hollywood

APPENDIX

Cowan-Kaufman-Morell Statement on Virus Isolation (SOVI)

Isolation: The action of isolating; the fact or condition of being isolated or standing alone; separation from other things or persons; solitariness
Oxford English Dictionary

The controversy over whether the SARS-CoV-2 virus has ever been isolated or purified continues. However, using the above definition, common sense, the laws of logic and the dictates of science, any unbiased person must come to the conclusion that the SARS-CoV-2 virus has never been isolated or purified. As a result, no confirmation of the virus' existence can be found. The logical, common sense, and scientific consequences of this fact are:

- the structure and composition of something not shown to exist can't be known, including the presence, structure, and function of any hypothetical spike or other proteins;
- the genetic sequence of something that has never been found can't be known;
- "variants" of something that hasn't been shown to exist can't be known;
- it's impossible to demonstrate that SARS-CoV-2 causes a disease called Covid-19.

In as concise terms as possible, here's the proper way to isolate, characterize and demonstrate a new virus. First, one takes samples (blood, sputum, secretions) from many people (e.g. 500) with symptoms which are unique and specific enough to characterize an illness. Without mixing these samples with ANY tissue or products that also contain genetic material, the virologist macerates, filters and ultracentrifuges i.e. *purifies* the specimen. This common virology technique, done for decades to isolate bacteriophages[1] and so-called giant viruses in every virology lab, then allows the virologist to demonstrate with electron microscopy thousands of identically sized and shaped particles. These particles are the isolated and purified virus.

These identical particles are then checked for uniformity by physical and/or microscopic techniques. Once the purity is determined, the particles may be further characterized. This would include examining the structure, morphology, and chemical composition of the particles. Next, their genetic makeup is characterized by extracting the genetic material directly from the purified particles and using genetic-sequencing techniques, such as Sanger sequencing, that have also been around for decades. Then one does an analysis to confirm that these uniform particles are exogenous (outside) in origin as a virus is conceptualized to be, and not the normal breakdown products of dead and dying tissues.[2] (As of May 2020, we know that virologists have no way to determine whether the particles they're seeing are viruses or just normal breakdown products of dead and dying tissues.)[3]

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1 Isolation, characterization and analysis of bacteriophages from the haloalkaline lake Elmenteita, KenyaJuliah Khayeli Akhwale et al, PLOS One, Published: April 25, 2019. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0215734 – accessed 2/15/21

2 "Extracellular Vesicles Derived From Apoptotic Cells: An Essential Link Between Death and Regeneration," Maojiao Li1 et al, Frontiers in Cell and Developmental Biology, 2020 October 2. https://www.frontiersin.org/articles/10.3389/fcell.2020.573511/full – accessed 2/15/21

3 "The Role of Extraellular Vesicles as Allies of HIV, HCV and SARS Viruses," Flavia Giannessi, et al, Viruses, 2020 May

If we have come this far then we have fully isolated, characterized, and genetically sequenced an exogenous virus particle. However, we still have to show it is causally related to a disease. This is carried out by exposing a group of healthy subjects (animals are usually used) to this isolated, purified virus in the manner in which the disease is thought to be transmitted. If the animals get sick with the same disease, as confirmed by clinical and autopsy findings, one has now shown that the virus actually causes a disease. This demonstrates infectivity and transmission of an infectious agent.

None of these steps has even been attempted with the SARS-CoV-2 virus, nor have all these steps been successfully performed for any so-called pathogenic virus. Our research indicates that a single study showing these steps does not exist in the medical literature.

Instead, since 1954, virologists have taken unpurified samples from a relatively few people, often less than ten, with a similar disease. They then minimally process this sample and inoculate this unpurified sample onto tissue culture containing usually four to six other types of material – all of which contain identical genetic material as to what is called a "virus." The tissue culture is starved and poisoned and naturally disintegrates into many types of particles, some of which contain genetic material. Against all common sense, logic, use of the English language and scientific integrity, this process is called "virus isolation." This brew containing fragments of genetic material from many sources is then subjected to genetic analysis, which then creates in a computer-simulation process the alleged sequence of the alleged virus, a so called in silico genome. At no time is an actual virus confirmed by electron microscopy. At no time is a genome extracted and sequenced from an actual virus. This is scientific fraud.

The observation that the unpurified specimen — inoculated onto tissue culture along with toxic antibiotics, bovine fetal tissue, amniotic fluid and other tissues — destroys the kidney tissue onto which it is inoculated is given as evidence of the virus' existence and pathogenicity. This is scientific fraud.

From now on, when anyone gives you a paper that suggests the SARS-CoV-2 virus has been isolated, please check the methods sections. If the researchers used Vero cells or any other culture method, you know that their process was not isolation. You will hear the following excuses for why actual isolation isn't done:

1. There were not enough virus particles found in samples from patients to analyze.

2. Viruses are intracellular parasites; they can't be found outside the cell in this manner.

If No. 1 is correct, and we can't find the virus in the sputum of sick people, then on what evidence do we think the virus is dangerous or even lethal? If No. 2 is correct, then how is the virus spread from person to person? We are told it emerges from the cell to infect others. Then why isn't it possible to find it?

Finally, questioning these virology techniques and conclusions is not some distraction or divisive issue. Shining the light on this truth is essential to stop this terrible fraud that humanity is confronting. For, as we now know, if the virus has never been isolated, sequenced or shown to cause illness, if the virus is imaginary, then why are we wearing masks, social distancing and putting the whole world into prison?

Finally, if pathogenic viruses don't exist, then what is going into those injectable devices erroneously called "vaccines," and what is their purpose? This scientific question is the most urgent and relevant one of our time.

We are correct. The SARS-CoV2 virus does not exist.

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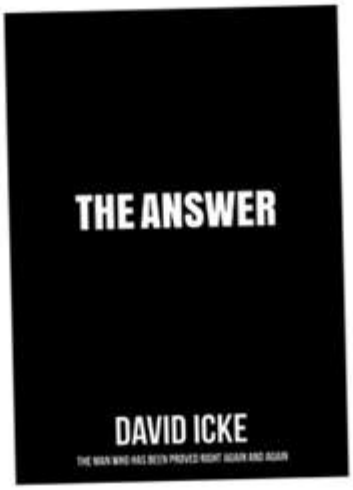

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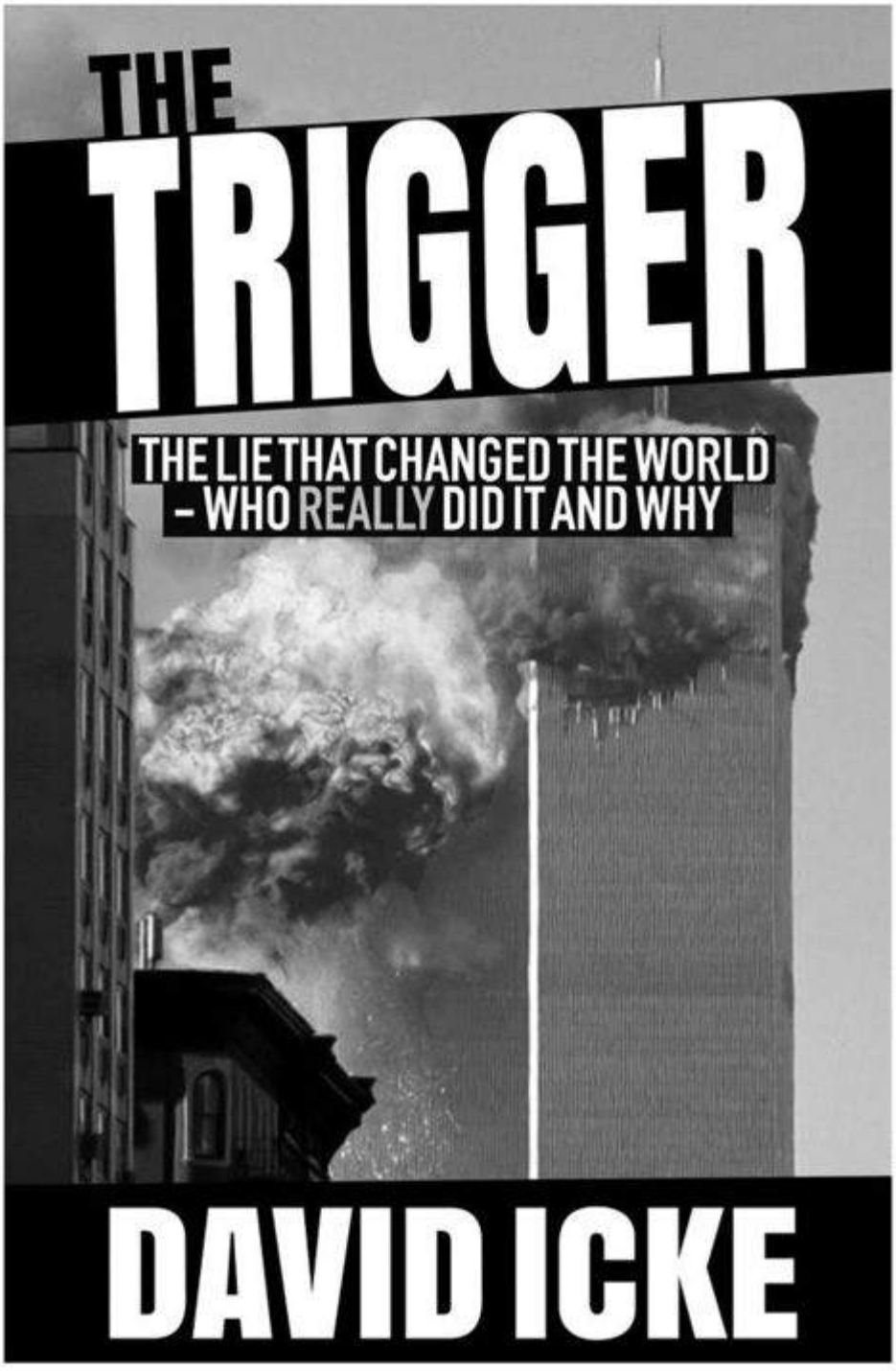
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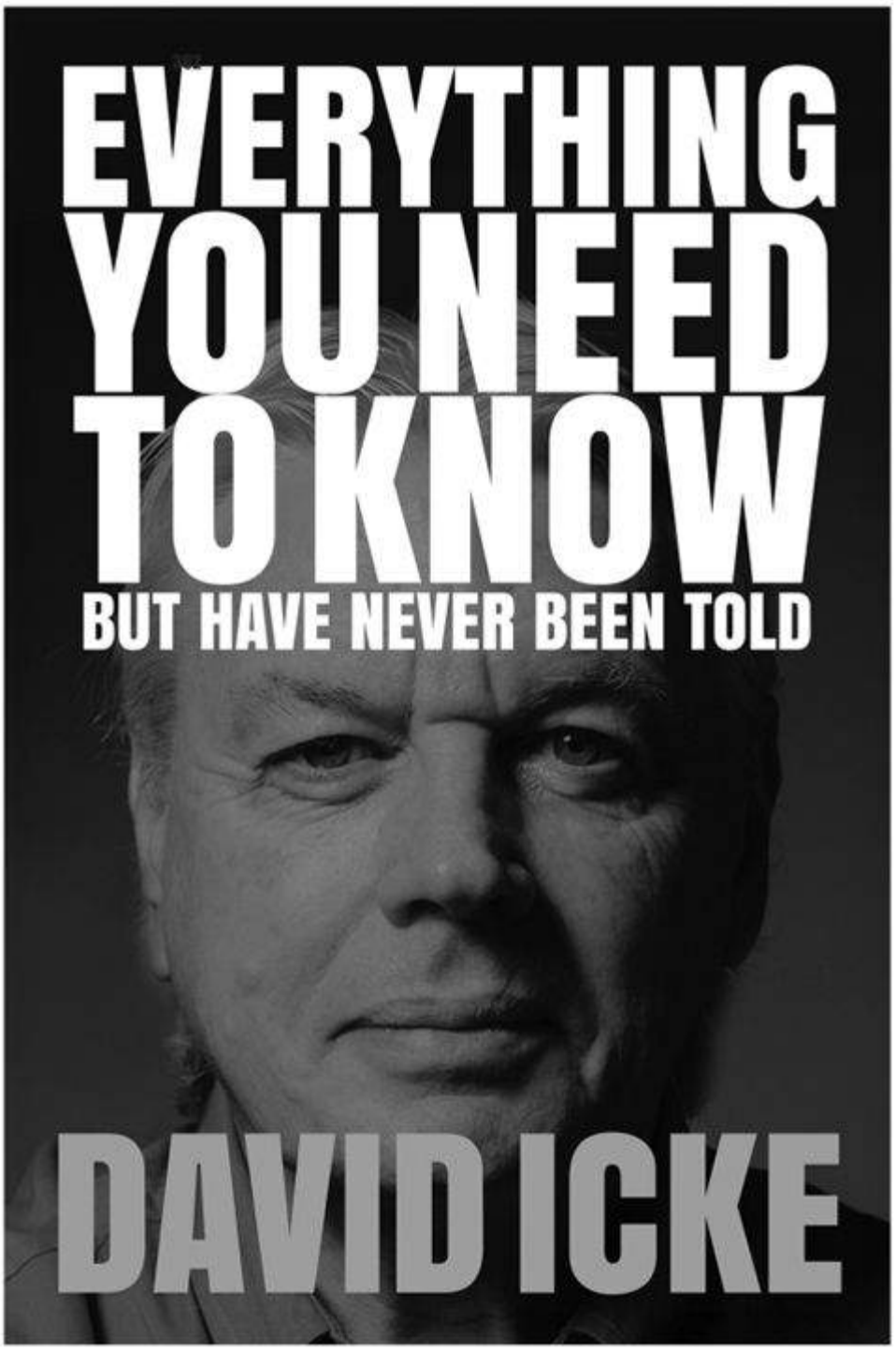
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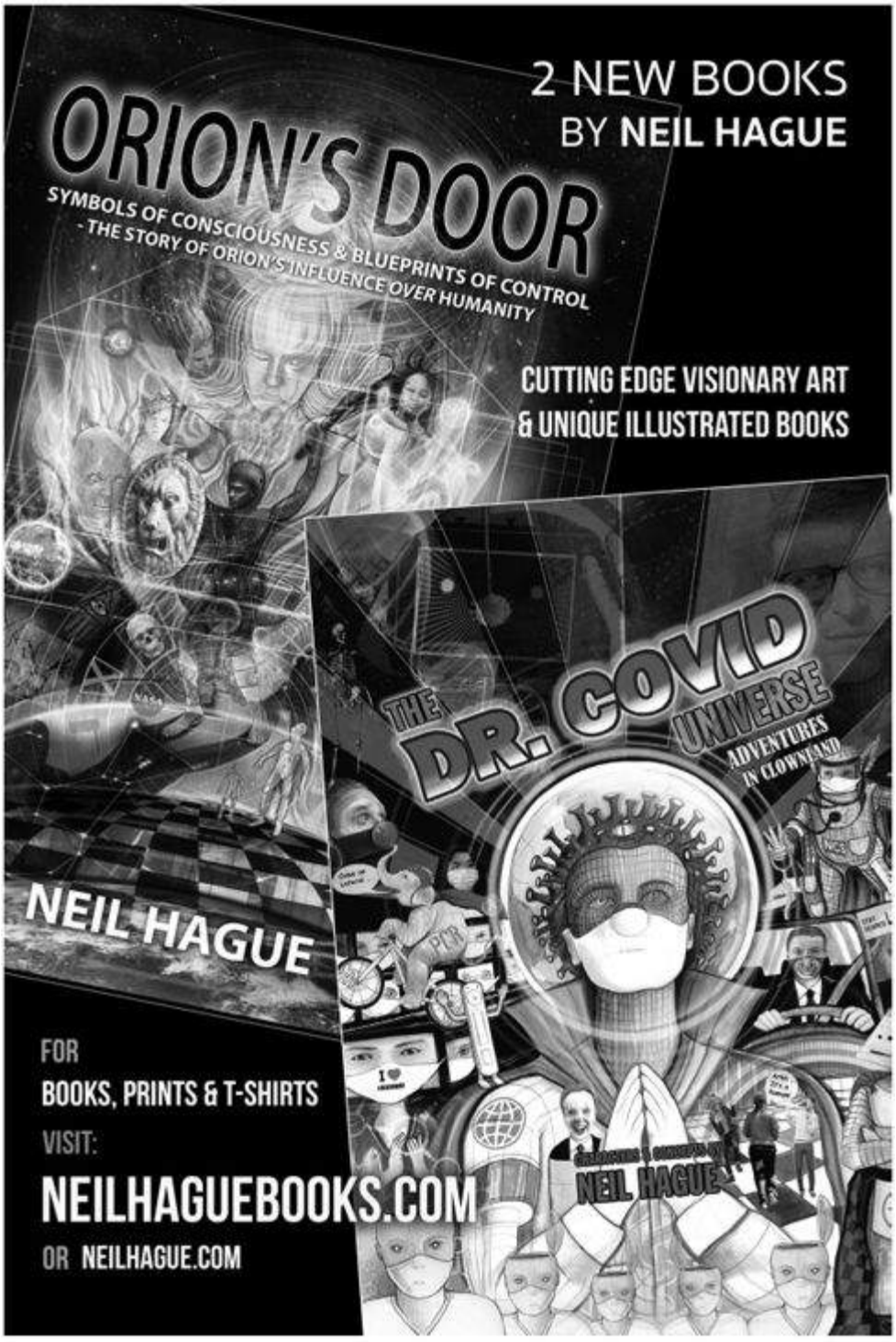
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